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From the Division of Food Chemistry, Royal Institute of Technology, Stockholm, Sweden

Studies on the Possibilities of Improving the Nutritive Value of Swedish Wheat Bread

II. The effect of supplementation with lysine, threonine, methionine, valine and tryptophan

By

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Abstract

ERICSON, L.-E. *Studies on the possibilities of improving the nutritive value of Swedish wheat bread. II.* Acta physiol. scand. 1960. *48.* 295—301. — A basal diet containing wheat bread as the source of protein and carbohydrate, and sufficient quantities of vitamins, essential fatty acids and minerals was supplemented with combinations of lysine, threonine, methionine, valine and tryptophan and fed to male weanling rats. The addition of lysine and threonine to the basal diet improved the nutritive value of the bread protein considerably more than the addition of lysine alone, as measured by the growth rate and the gain in weight per g of nitrogen consumed. On a bread diet containing 2.0 % nitrogen and fortified with 0.32 % L-lysine and 0.2 % DL-threonine, the rats gained 21.0 g per g of nitrogen consumed. The corresponding figures for rats given isonitrogenous diets in which spray dried skim milk or egg albumin supplied the protein were 20.1 and 22.4. No further improvement was obtained on addition of methionine, valine and tryptophan to bread diets supplemented with lysine and threonine.

In a previous paper (ERICSON and OVENFORS 1959) we reported that supplementation with lysine increases the protein value of Swedish wheat bread. However, the effect of lysine supplementation, as measured by the growth of weanling albino rats, was less than would be expected from data reported by other authors. This suggested that Swedish wheat bread (prepared with 3.3 %

roller dried skim milk) is deficient not only in lysine but also in other amino acids essential for the growth of rats. British research workers have recently reported that not only lysine but also threonine (BENDER 1958, HUTCHINSON, MORAN and PACE 1958) and methionine (BENDER 1958) must be added to wheat bread diets in order to obtain optimal utilization of the bread protein for the rat. Wheat flour has been reported to be deficient also in valine (SURE 1953). In view of these and of other observations a study was made on the growth of weanling rats given Swedish wheat bread supplemented with lysine, threonine, methionine, valine and tryptophan.

Experimental

Four experiments were carried out. For the first three experiments male weanling rats of the Wistar strain were purchased from Søborg Dyrefarm, Copenhagen. They were housed individually in glass containers having a diameter of 20 cm and containing wood shavings. The experiments took place at the Department of Physiology, Royal Veterinary College, Stockholm. In the fourth experiment male weanling rats of the Wistar strain, bred at the National Institute of Public Health, Stockholm, were used. These rats were housed separately in wire bottom cages during the experiment which was carried out at the National Institute of Public Health.

The animals were fed the diets *ad libitum*.

The diets. All bread diets contained 90 % air dried wheat bread, 3 % of a salt mixture, 4.5 % soya bean oil, 0.2 % cod liver oil and a vitamin mixture. The same amounts of the salt and vitamin mixtures and of the oils were used for diets prepared with egg albumin or spray dried skim milk (fourth experiment). The two latter diets had the same nitrogen content as the bread diets and contained wheat starch as the carbohydrate source.

The salt mixture was that of HEGSTED *et al.* (1941). The vitamin mixture (HARPER *et al.* 1953) supplied in mg per 100 g ration: thiamine · HCl 0.5, riboflavin 0.5, niacin 1.0, calcium pantothenate 2.0, pyridoxine 0.25, biotin 0.01, pteroylglutamic acid 0.02, cyanocobalamin 0.002, inositol 10, menadione 0.5 and choline chloride 150. The cod liver oil provided approximatively 150 I. E. vitamin A and 15 I. E. vitamin D per 100 g of ration.

The bread used in the first two experiments had been prepared according to the formula:

water	1 liter
wheat flour (70 % extraction)	1.8 kg
lard	40 g
roller dried skim milk (< 1 % fat)	100 g
sugar	20 g
salt	10 g
malt extract	20 g
yeast	70 g/liter dough

The bread used in experiments three and four had been baked from a dough with the composition:

water	1 liter
wheat flour (70 % extraction)	1.8 kg
lard	28 g

roller dried skim milk (< 1 % fat)	100	g
sugar	20	g
salt	30	g
malt flour	5	g
yeast	65	g/liter dough

The bread was cut in cubes approximately 2 × 2 × 2 cm and dried over night in a stream of air having a temperature of 20° C. The dried bread contained 91 % dry matter. It was ground to a powder in an Electrolux "Assistent" bread mill.

The nitrogen content of the nonsupplemented bread diets was 1.90 % and the lysine content 2.5 g per 16 g N. The nitrogen content of the bread diets fortified with amino acids was in each experiment adjusted to the same level by the addition of glycine.

Analytical procedures. The nitrogen determinations were made according to a Kjeldahl method (PERRIN 1953). The ammonia was collected in boric acid and titrated with 0.01 N HCl.

To determine the dry weight the samples were heated at 104° C for 20 h.

Lysine analyses were made microbiologically using *Leuconostoc mesenteroides* P-60 in the medium of STEEL *et al.* (1949). The samples were hydrolysed in 6N HCl at 104° C for 24 h.

Results

In the first experiment one group of rats was given the basal bread diet supplemented with 0.8 % L-lysine·HCl and another group was given the basal bread diet supplemented with 0.8 % L-lysine·HCl and 0.5 % DL-threonine, 0.5 % DL-methionine and 0.5 % DL-valine. Each group contained 10 animals which were fed the diets for 21 days. The average growth rates of the two groups are shown in Fig. 1. It is obvious that the addition of threonine, methionine and valine improved the biological value of the lysine supplemented bread.

The second experiment comprised 5 groups of rats with 6 rats in each group. The different groups received the basal bread diet supplemented with:

(1) 0.8 % L-lysine·HCl;
(2) » + 0.5 % DL-methionine;
(3) » + 0.5 % DL-threonine;
(4) » + 0.5 % DL-methionine + » ;
(5) » + » + » + 0.5 % DL-valine.

The diets were fed over a period of 25 days.

Fig. 2 gives the average growth curves for the rats given these diets. It can be seen that the addition of lysine and methionine (diet 2) to the bread diet gave no better growth than the addition of lysine alone (diet 1). Rats receiving the basal bread diet supplemented with lysine and threonine (diet 3), on the other hand, grew almost twice as fast as the rats receiving the diet supplemented only with lysine. No further improvement of the diet fortified with lysine and threonine could be obtained by the addition of methionine (diet 4) or methio-

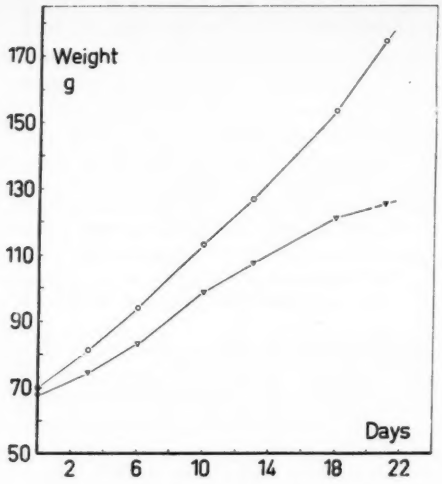

Fig. 1. Growth curves for the rats in the first experiment.
▽—▽ Bread diet supplemented with 0.8 % L-lysine · HCl.
○—○ Bread diet supplemented with 0.8 % L-lysine · HCl, 0.5 % DL-threonine, 0.5 % DL-methionine and 0.5 % DL-valine. The standard error of the mean, referring to the final weights, was ± 4 for both groups.

nine plus valine (diet 5). Addition of 0.5 % DL-tryptophan (at the arrow in Fig. 2) to the diet already supplemented with lysine, threonine, methionine and valine decreased the growth rate. This experiment thus showed that the wheat bread used is deficient in both lysine and threonine, but not in methionine, valine or tryptophan.

In the third experiment, 5 groups of rats were fed bread diets to which varying amounts of lysine and threonine had been added (see Table I). Six rats were used in each group and they were fed the diets for 23 days. Unfortunately the weights of the rats obtained for this experiment varied considerably and the animals appeared to be in a bad physical condition at the beginning of the experiment, as judged by their poor appetite and initially slow gain in weight. The average growth rate was therefore less than in previous experiments. However, the data (Table I) indicate that supplementation of the bread diet with 0.4 % L-lysine · HCl and 0.2 % DL-threonine, stimulates equally good growth as does supplementation with 0.8 % L-lysine · HCl and 0.5 % DL-threonine. In accordance with earlier observations (ERICSON and OVENFORS 1959) it was noted that addition of 0.4 % L-lysine · HCl to the basal bread diet was sufficient to satisfy the lysine requirements of the growing rat.

The object of the fourth experiment was to verify the results of the previous experiments under different conditions, and to compare the nutritive values

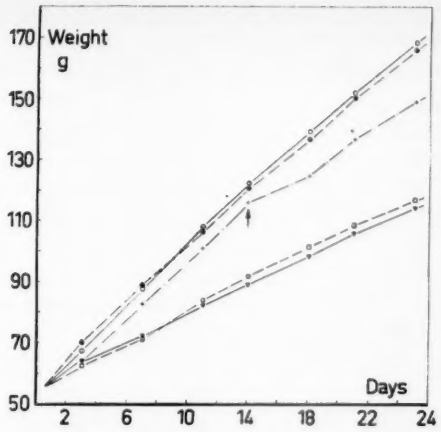

Fig. 2. Growth curves for the rats in the second experiment.

▽—▽ Basal bread diet + 0.8 % L-lysine · HCl.
□—□ » » » + » + 0.5 % DL-methionine.
○—○ » » » + » + 0.5 % DL-threonine.
●—● » » » + » + 0.5 % DL-methionine + + 0.5 % DL-threonine.
×—× » » » + » + 0.5 % DL-methionine + + 0.5 % DL-threonine + 0.5 % DL-valine. DL-tryptophan (0.5 %) added at the arrow.

The standard error of the mean, referring to the final weights, was ± 5, ± 8, ± 8, ± 4 and ± 6 respectively for the five groups.

of nonsupplemented and supplemented bread protein, spray dried skim milk and egg albumin. Five groups of rats with 10 rats in each group were given the diets listed in Table II for a period of 24 days. Weight gain and food consumption were recorded for each animal.

Table I. Composition of diets and average gain in grams per day of the groups of rats used in the third experiment

Group no.	Basal bread diet supplemented with	Average gain in weight, g/day
1	0.4 % L-lysine · HCl	2.4 ± 0.3[1]
2	0.4 % L-lysine · HCl + 0.2 % DL-threonine	3.8 ± 0.3
3	0.4 % L-lysine · HCl + 0.5 % DL-threonine	3.6 ± 0.2
4	0.8 % L-lysine · HCl + 0.2 % DL-threonine	3.9 ± 0.2
5	0.8 % L-lysine · HCl + 0.5 % DL-threonine	3.6 ± 0.3

[1] Standard error of the mean.

Table II. Composition of diets and average gain in weight per day and per g of nitrogen consumed of the rats in the fourth experiment

Group no.	Diets	Nitrogen content %	Average gain in weight g/day	Average gain in weight per g N consumed g/g
1	Basal bread diet (+ glycine)	1.96	1.36 ± 0.21[1]	9.82 ± 0.72[1]
2	» » » + 0.4 % L-lysine · HCl	2.00	4.27 ± 0.34	18.2 ± 0.61
3	» » » + » + + 0.2 % DL-threonine	2.00	5.78 ± 0.27	21.0 ± 0.29
4	Spray dried skim milk (33.2 %).....	2.06	4.60 ± 0.21	20.1 ± 0.31
5	Egg albumin (14.6 %).....	1.97	4.10 ± 0.14	22.4 ± 0.31

[1] Standard error of the mean.

Table II summarizes the results. The rats given the bread diet supplemented with L-lysine grew more than three times as fast as those given the nonsupplemented diet. A considerable further increase in the growth rate was again observed when threonine (group 3) was added to the diet already supplemented with lysine. The growth rate of group 3 was also significantly higher than that of the group receiving the skim milk diet or of the group given the diet containing egg albumin. However, if the gains in weight per g of nitrogen consumed are compared, it is evident that egg albumin was a slightly better source of protein than wheat bread fortified with lysine and threonine. The latter diet was superior to the milk diet. The difference between the average gain in weight per g of nitrogen consumed was significant at the 0.1 % level for the groups 2 and 3, at the 0.5 % level for the groups 3 and 4, and at the 5 % level for the groups 3 and 5.

Discussion

The results show that the wheat bread used in the present experiments is deficient both in lysine and threonine. This was expected in view of the results recently published by BENDER (1958) and by HUTCHINSON, MORAN and PACE (1958). ROSENBERG, ROHDENBURG and BALDINI (1954), however, failed to obtain any beneficial effect from threonine supplementation of bread diets fortified with lysine and having the same nitrogen content as ours. The protein efficiency ratios obtained by these authors were — as already pointed out by BENDER (1958) — conspicuously low. The highest value reported by them was 2.43, whereas we observed a protein efficiency ratio of 3.36 for the bread diet fortified with lysine and threonine and 3.60 for the diet containing egg albumin. HUTCHINSON, MORAN and PACE (1958) found that rats fed a bread diet containing 2.31 % nitrogen and supplemented with 0.5 % L-lysine and 0.2 % L-threonine gained 18.8 g per g of nitrogen consumed. This figure

agrees fairly well with our value of 21.0, considering that our diets had a lower content of nitrogen (2.0 %).

In contrast to the observations of BENDER (1958), we obtained no further improvement by the addition of methionine to a bread diet containing lysine and threonine. It is possible that this depends on the fact that the diets used by BENDER contained less nitrogen (1.5 %) than ours (2.0 %). It is also conceivable that the large amount of threonine — up to seven times the quantity needed — that BENDER added to the bread diets caused an amino acid imbalance that was counteracted by methionine. Our results also show that the concentrations of valine and tryptophan in the wheat bread used by us are sufficient to meet the requirements of the growing rat. Wheat flour, on the other hand, appears to be deficient in valine (SURE 1953).

The data of HUTCHINSON, MORAN and PACE (1958) indicate that the protein efficiency ratio of wheat bread fortified with lysine and threonine is approximatively 80 % of that of whole egg protein. In our experiments the bread diets to which lysine and threonine were added had a protein efficiency ratio that was more than 90 % of that of egg albumin.

The author would like to express his gratitute to Prof. Y. ZOTTERMAN for making the animal quarters at the Department of Physiology, Royal Veterinary College, Stockholm, available for the first three experiments reported here, and to Profs. E. BRUNIUS and E. ABRAMSON, National Institute of Public Health, Stockholm, for permitting the fourth experiment to be carried out at the latter Institute. The assistance of I. FRÖLICH and G. LID and the financial support of the Swedish National Science Research Council and the Knut and Alice Wallenberg's Foundation is also gratefully acknowledged.

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Histamine and a Lipid-Soluble Smooth-Muscle Stimulating Principle ('SRS') in Anaphylactic Reaction

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Abstract

CHAKRAVARTY, N. and B. UVNÄS. *Histamine and a lipid-soluble smooth-muscle stimulating principle ('SRS') in anaphylactic reaction.* Acta physiol. scand. 1960. *48.* 302—314. — The relationship between the occurrence of histamine and 'SRS' in anaphylactic reaction was investigated in guinea-pig tissue. The temperature and pH curves for histamine and 'SRS' were similar. Anoxia and lack of calcium inhibited equally the appearance of both. When histamine release was inhibited by polymeric enzyme inhibitors or by an organic dye, there was concomitant inhibition of 'SRS'. Moreover, enzyme inhibitors which react with sulphydryl or amino groups — iodoacetate, allicin, 2:4-dinitrofluorobenzene, phenylisocyanate and acetic anhydride — blocked both histamine and 'SRS'. Glutathione and cysteine partly reversed the inhibitory effect of iodoacetate on both histamine and 'SRS'. While histamine is released from a loose binding in the tissue, 'SRS' seems to be formed during anaphylactic reaction. The observations may be explained by postulating that antigen-antibody reaction in anaphylaxis activates an enzyme system, probably with essential sulphydryl and amino groups in the mast cells, and results in the appearance of both histamine and 'SRS'.

In a separate communication we have reported the appearance of a lipid-soluble, smooth-muscle stimulating principle ('SRS') from sensitized guinea-pig lungs following anaphylactic reaction *in vitro* (CHAKRAVARTY 1960 b).

In the course of that investigation a striking correlation was observed between the occurrence of histamine and of 'SRS'. It was thought that the appearance of the two substances might depend on the same or similar mechanisms. In the present investigation the correlation was found to extend over a wide range of experimental conditions. The findings could be explained by the hypothesis that the same enzymatic mechanism in the mast cell leads to the appearance of both substances. While histamine is released from a loose binding, 'SRS' seems to be formed in the process of the anaphylactic reaction.

Material and Methods

Guinea pigs sensitized with egg albumin were used to produce anaphylactic reaction in lung pieces *in vitro*. The method of sensitization and details of the experimental procedure have been described elsewhere (CHAKRAVARTY 1960 a, 1960 b). The antigen (1 mg/ml) was kept in contact with the sensitized tissue in Tyrode solution at 37° C for 10 min. When an inhibitor was used the tissues were incubated with it in Tyrode solution for 15—20 min before addition of the antigen. Some modification of the method was necessary to determine the effects of temperature, pH, etc. These will be referred to in the relevant sections. The pH was determined electrometrically. In certain experiments in which successive values for histamine and 'SRS' were taken for different periods of time, a small amount of the incubation fluid was withdrawn each time until the completion of the experiment.

Histamine was assayed on atropinized (atropine sulphate 1.5×10^{-6} M) guinea-pig ileum. The values are expressed as histamine base.

'SRS' was also assayed on guinea-pig ileum in the presence of atropine (1.5×10^{-6} M) and an antihistaminic (mepyramine 10^{-7} M to 10^{-6} M), the quantitative values for 'SRS' being expressed in units. Details of the method of 'SRS' assay have been published (CHAKRAVARTY 1959). 'SRS' was tested either in the original incubation fluid or following extraction in alcohol and ether. The extraction procedures have been described previously (CHAKRAVARTY, HÖGBERG and UVNÄS 1959, CHAKRAVARTY 1960 b).

Some of the inhibitors and reactivators influenced the contraction of the ileum, especially in higher concentrations. Whenever they were present in the test solution, the corresponding amounts were added to the standard histamine and 'SRS'. In those experiments in which alcohol was used as an inhibitor the samples were freeze-dried and redissolved in water before testing. When allicin was used as an inhibitor the samples were heated in a boiling water bath for 10 min to avoid interference with the gut.

Hip-seed polysaccharide, used as one of the inhibitors, was prepared from air dried hips by the method described by HÖGBERG *et al.* (1957). Allicin ($C_6H_{10}S_2O$) was extracted from garlic (*Allium sativum*) by the method of CAVALLITO and BAILEY (1944). Pk 312, which is the code name for a formaldehyde condensation product of o-aminobenzoic acid, was supplied by AB Leo, Hälsingborg, Sweden.

Results

Effect of Temperature

The effect of temperature on the appearance of histamine and 'SRS' in anaphylactic reaction was investigated in three experiments. The lung pieces were incubated at different temperatures for 5—10 min before addition of

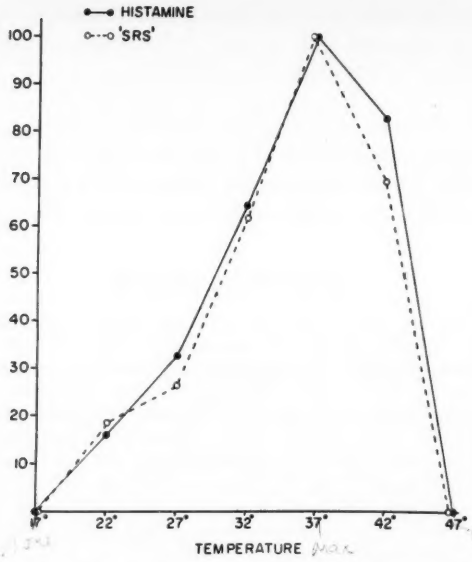

Fig. 1. Effect of temperature (°C) on the appearance of histamine and 'SRS' in anaphylactic reaction. The highest values for both histamine and 'SRS' are shown as 100.

antigen, and incubation was continued for another 10 min at the respective temperatures. Fig. 1 illustrates the temperature curve in one experiment, the values being expressed in relative proportions taking the maximal values for both histamine and 'SRS' (2.8 μg/g and 220 units/g respectively) as 100. The maximal amounts of histamine and 'SRS' appeared at 37° C; the values for both at 42° C were only slightly less; and neither histamine nor 'SRS' appeared at 47° C. At lower temperatures both substances rose more gradually between 17° C and 37° C. The correlation between histamine and 'SRS' at all temperatures is so striking that the results could well be comprised in one curve. The results in two other experiments were similar. It may be noted that at lower temperatures there is no irreversible change and if tissues are brought up to 37° C from a lower temperature before the addition of antigen, the occurrence of neither histamine nor 'SRS' is affected. On the other hand at temperatures of 45° C or more, an irreversible change occurs in the tissue, preventing the appearance of both histamine and 'SRS'. This will be evident from the following experiment: On incubating one of two uniform samples of tissue from sensitized guinea-pig with antigen directly at 37° C, 2.3 μg/g histamine and 256 units/g 'SRS' were found in the incubation fluid. But when the other sample was heated to 45° C for 10 min prior to incubation

Table I. Effect of pH on the appearance of histamine and 'SRS'

Expt.	pH	Histamine (μg/g)	'SRS' (units/g)
1	5.25	0	0
	5.73	0.18	0
	6.30	0.85	13
	6.70	1.5	26
	7.65	1.8	141
	8.84	[1]1.4	226
2	5.83	0.76	0
	6.72	4.1	80
	8.02	3.6	306
	9.15	2.4	163
3	5.60	0	0
	6.30	0.64	0
	7.50	0.87	43
	7.75	0.83	49
	8.50	0.82	112
	9.50	0	5
	9.98	0	0

[1] This includes some spontaneous release.

with antigen at 37° C, histamine and 'SRS' values were reduced to 0.11 μg/g and 17 units/g respectively; *i. e.* 5 to 7 % of the control values.

Effect of pH

The effect of pH on the appearance of histamine and of 'SRS' in anaphylaxis is shown in Table I. The tissues were suspended in 0.85 per cent sodium chloride solution containing 1.8×10^{-3} M calcium chloride and buffered with veronal buffer at different pH. A few drops of dilute NaOH or HCl (0.1 to 1 N) were added to the buffer solution, when necessary, to obtain the desired pH range. The pieces of lung were washed at 37° C for 25 min at different pH before incubation with antigen in the respective solutions. The pH was again taken at the end of the experiment, and only the final pH is shown. The complete pH curve could be elucidated after taking into consideration the spontaneous release of histamine that occurs with extreme changes in pH. 'SRS' was not released spontaneously at acid pH (up to 4). At alkaline pH above 8.5, in only two out of five experiments was spontaneous histamine release associated with the liberation of small amounts of a substance producing slow contraction of guinea-pig ileum. In these two cases the control values, estimated in 'SRS' units, were deducted to determine the effect of pH in anaphylaxis on the

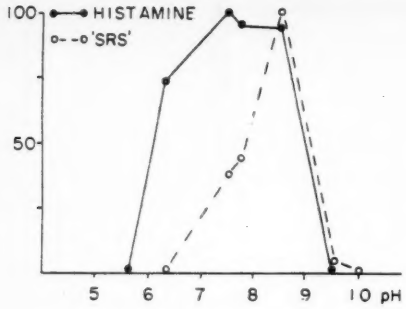

Fig. 2. Effect of pH on the appearance of histamine and 'SRS' in anaphylactic reaction. The highest values for both histamine and 'SRS' are shown as 100.

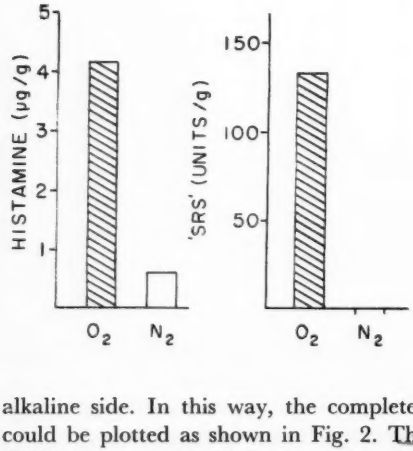

Fig. 3. Effect of anoxia on the appearance of histamine and 'SRS' in anaphylactic reaction. Anoxia was produced by nitrogen saturation of Tyrode solution in which the tissues were suspended.

alkaline side. In this way, the complete pH curve for histamine and 'SRS' could be plotted as shown in Fig. 2. The appearance of both histamine and 'SRS' ceased at pH 5.6 to 6.3 on the acid side, and 9.5 to 10 on the alkaline side. The optimal pH for histamine release was 7 to 8.5; for 'SRS' it was within the same range but had a sharper peak at about 8.5. Indeed, if the 'SRS' values in the incubation fluid of these experiments truly reflect the amounts of 'SRS' formed in the tissues (see below) the results might suggest a slightly higher pH optimal for 'SRS' than for histamine (see Table I).

Effect of Anoxia

We have reported elsewhere that oxygen is necessary for anaphylactic histamine release in guinea-pig but not in rat (CHAKRAVARTY 1960 a). It was of interest to ascertain whether the appearance of histamine and of 'SRS' in anaphylactic reaction in guinea-pig were equally dependent on oxygen. Lung pieces from sensitized guinea-pigs were incubated with antigen in oxygenated and oxygen-free (nitrogen saturated) Tyrode solution. In oxygenated Tyrode solution, as shown in Fig. 3, 4.2 µg/g histamine and 133 units/g

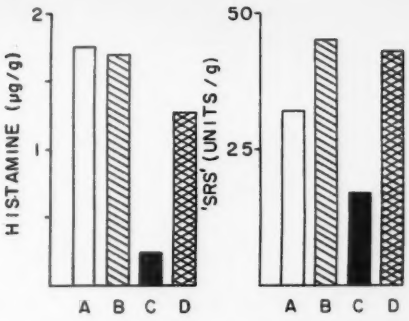

Fig. 4. Effect of calcium lack on the appearance of histamine and 'SRS' in anaphylactic reaction.
A = 0.85 % NaCl + Veronal buffer + 1.8×10^{-3} M $CaCl_2$
B = 0.85 % NaCl + Veronal buffer
C = 0.85 % NaCl + Veronal buffer + 3.4×10^{-4} M EDTA
D = Same as C but 10^{-2} M $CaCl_2$ added later.

'SRS' were found; but in oxygen-free Tyrode solution only 0.61 µg/g histamine (*i. e.*, 15 per cent of the amount in the presence of oxygen) was released and no 'SRS' could be detected. Considering the sensitivity of the test bath to 'SRS' it could be concluded that anoxia was equally effective in blocking histamine and 'SRS'. The result was similar in a second experiment.

Effect of Calcium Lack

It has been shown previously that calcium is necessary for anaphylactic histamine release (HUMPHREY and JAQUES 1955, MONGAR and SCHILD 1958, CHAKRAVARTY 1960 a). To study the effect of calcium ions on the occurrence of histamine and 'SRS' in anaphylactic reaction, lung pieces from sensitized guinea-pigs were suspended in 0.85 per cent NaCl buffered with veronal or phosphate buffer to give a pH of about 8. When tissues were suspended in this solution with 1.8×10^{-3} M calcium chloride, the release of histamine was only slightly lower than in Tyrode solution. As will be seen in Fig. 4, however, the amounts of histamine and 'SRS' in the incubation fluid were scarcely affected by the addition of calcium chloride to physiological saline with veronal buffer. The tissue calcium was apparently sufficient for the anaphylactic reaction to proceed normally. If, however, 3.4×10^{-4} M ethylenediaminetetraacetic acid (EDTA), a chelating agent, was added to the calcium-free solution to bind the tissue calcium, the appearance of both histamine and 'SRS' was appreciably inhibited. EDTA, it is true, will bind other metal ions. However, the specific role of calcium ions in these reactions is indicated by the observation that when calcium chloride ($CaCl_2$ 10^{-2}M) was added 10 min after antigen, both histamine and 'SRS' values were restored almost completely in the ensuing 10 minutes. Addition of an equimolar amount of magnesium ions did not affect the release of either histamine or 'SRS'. In other experiments where phosphate buffer was used instead of veronal buffer, the appearance of histamine and 'SRS' was completely inhibited not only by EDTA, but also by washing

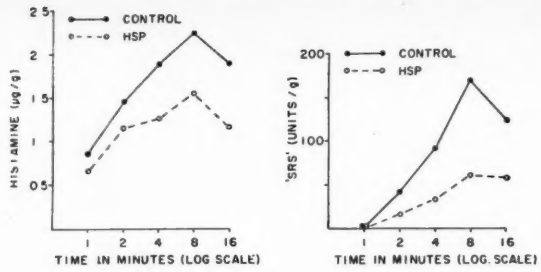

Fig. 5. Inhibition of anaphylactic reaction by hip seed polysaccharide (HSP). Note that the appearance of both histamine and 'SRS' is inhibited.

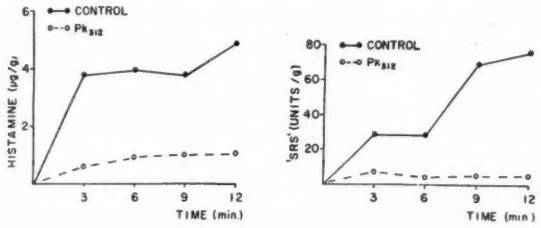

Fig. 6. Inhibition of anaphylactic reaction by Pk 312 (formaldehyde condensation product of o-aminobenzoic acid). Histamine was tested in the Tyrode solution and 'SRS' following ether extraction.

the tissue in a large volume of buffered physiological saline prior to incubation with antigen in fresh solution. Subsequent addition of calcium chloride completely restored the histamine and 'SRS' values in the ensuing ten-minute period.

Effect of Enzyme Inhibitors

If the appearance of both histamine and 'SRS' is mediated by the same enzymatic process, as seems probable from the preceding observations, an inhibition of histamine release in anaphylaxis should be accompanied by inhibition of the occurrence of 'SRS'. We have reported elsewhere the blocking effect of enzyme inhibitors on mast cell disruption and histamine release in anaphylactic reaction (HÖGBERG and UVNÄS 1958, 1959, CHAKRAVARTY 1960 a). Some of these inhibitors have been investigated for possible effects on the appearance of 'SRS'.

Hip-seed polysaccharide (HSP) is an effective inhibitor of histamine release (HÖGBERG *et al.* 1957). The experiment illustrated in Fig. 5 shows that con-

Table II. Inhibition of the appearance of histamine and 'SRS' in anaphylactic reaction

Inhibitor	Conc. of inhibitor	Histamine μg/g		'SRS' units/g		% inhibition	
		Without inhibitor	With inhibitor	Without inhibitor	With inhibitor	H	'SRS'
Allicin	10^{-3} M	1.2	0.14	65	0	88	100
	10^{-3} M	1.2	0.15	109	11	87	90
	10^{-3} M	6.6	0.91	373	0	86	100
	7×10^{-4} M	4.2	1.1	132	40	74	70
	7×10^{-5} M	4.2	4.8	132	170	0	0
Iodoacetate	10^{-2} M	3.7	0.15	850	0	96	100
	10^{-3} M	3.7	0.83	850	178	78	79
	10^{-3} M	1.3	0.16	115	31	88	73
	10^{-3} M	2.1	0.35	162	26	83	84
	10^{-3} M	7.6	0.24	513	0	97	100
	5×10^{-4} M	2.1	0.45	162	62	79	62
	10^{-4} M	3.7	2.9	850	1,063	22	0
Phenylisocyanate	10^{-3} M	7.6	4.2	513	299	45	42
Acetic anhydride	[1] 10^{-2} M	1.9	0	50	0	100	100
	[1] 10^{-2} M	3.5	0.10	75	0	97	100
	10^{-3} M	7.6	6.2	513	345	18	33
Dinitrofluorobenzene	[1] 10^{-3} M	1.9	0	50	0	100	100
	10^{-4} M	3.2	1.0	444	28	69	94
Ethyl alcohol	2×10^{-2} v/v	2.4	0.21	468	5	91	99
	10^{-2} v/v	3.1	1.1	144	53	65	63
	10^{-2} v/v	2.4	0.94	468	82	61	82
	10^{-2} v/v	2.9	0.95	180	27	67	85
	10^{-3} v/v	2.4	2.7	468	468	0	0
Rhodamine B	[1] 10^{-3} M	2.5	0.22	361	0	91	100
	[1] 10^{-3} M	2.4	0.33	179	0	86	100

[1] Tissue was washed after incubation with inhibitor.

comitant with the inhibition of histamine release in anaphylactic reaction HSP inhibits the appearance of 'SRS'. Another polymeric enzyme inhibitor, Pk 312 (see p. 303) caused, after 12 min incubation with antigen, 78 and 93 % inhibition of the appearance of histamine and 'SRS' respectively (see Fig. 6). The results obtained with seven other inhibitors are presented in Table II. Allicin and iodoacetate in concentrations of 5×10^{-4} M to 10^{-3} M caused more than 70 % inhibition of histamine release with a more or less proportional inhibition of 'SRS'. 2: 4-dinitrofluorobenzene (DNFB) in a concentration of 10^{-4} M was also effective in inhibiting the occurrence of both histamine and 'SRS', as was acetic anhydride in concentration of 10^{-3} M. The effect of the last two inhibitors persisted even when the tissue was washed following in-

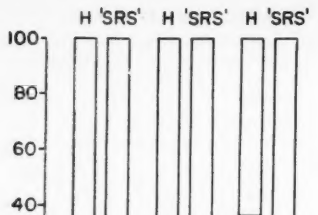

Fig. 7. Inhibition of anaphylactic reaction: correlation between histamine and 'SRS'. Histamine and 'SRS' values in inhibited reaction are shown as a percentage of the corresponding control values (without inhibitor). Mean values from 3 experiments are shown for each inhibitor. Concentrations: allicin: 7×10^{-4} M, 10^{-3} M, 10^{-3} M; iodoacetate: 10^{-3} M; ethyl alcohol: 10^{-2} v/v (1.7×10^{-1} M).

cubation with inhibitors and, resuspended in Tyrode solution before the addition of antigen. Phenylisocyanate (10^{-3} M) caused 40 to 45 % inhibition of the appearance of histamine and 'SRS'.

Since ethyl alcohol and the triphenylmethane dye, rhodamine B, had been found to be effective inhibitors of anaphylactic histamine release in guinea pig (CHAKRAVARTY 1960 a), their effect on the occurrence of 'SRS' was also investigated. Ethyl alcohol in a concentration of 10^{-2} v/v, produced over 60 % inhibition of the occurrence of both histamine and 'SRS'. Rhodamine B, in a concentration of 10^{-3} M, caused 85 to 100 % inhibition in the appearance of histamine and 'SRS', and the effect persisted even after the inhibitor was washed out prior to addition of antigen. The action of three of the last seven inhibitors — allicin, iodoacetate and ethyl alcohol — has been illustrated in Fig. 7.

The polymeric substances, hip-seed polysaccharide and Pk 312, are non-specific enzyme inhibitors. But allicin and iodoacetate are more or less specific inhibitors of sulphydryl enzymes. The evidence that enzymes with sulphydryl groups participate in the anaphylactic reaction and lead to the appearance of both histamine and 'SRS', finds further support in the observation that the inhibitory action of iodoacetate on the occurrence of histamine and 'SRS' could be partially reversed by pretreatment of the tissues with glutathione or cysteine. The tissues were incubated with four to eight times higher molar concentrations of glutathione or cysteine for 15 min before addition of the inhibitor (iodoacetate, 5×10^{-4} M or 10^{-3} M). After incubation for further 15 min, antigen was added. In all cases preincubation of the tissues with reactivators brought about an increase in the histamine and 'SRS' values, as compared to those produced by the inhibitor alone. The mean effect of two experiments with 10^{-3} M iodoacetate and with glutathione as reactivator is illustrated in Fig. 8.

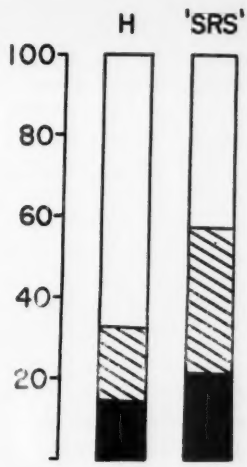

Fig. 8. Inhibition of the appearance of histamine and 'SRS' in anaphylactic reaction by iodoacetate and its partial reversal by reduced glutathione. The data are presented taking the control values (without inhibitor) as 100. The solid block indicates the inhibited reaction due to iodoacetate (10^{-3} M) and the solid plus shaded blocks show the values in presence of iodoacetate (10^{-3} M) and glutathione (4×10^{-3} M & 8×10^{-3} M). The results are the mean values from two experiments.

Of the other inhibitors, acetic anhydride has been shown by HÖGBERG and UVNÄS (1957) to react specifically, in their experiments, by acetylation of the amino group. DNFB and phenylisocyanate also combine with amino groups, though other reactions are possible.

The question still remains whether 'SRS' is released from a loose binding, as in the case of histamine or is formed during anaphylactic reaction (KELLAWAY and TRETHEWIE 1940, BROCKLEHURST 1956). Table III shows the results of two of our experiments designed to answer this question. When pieces of guinea-pig lungs were extracted with 80 per cent alcohol directly, most of the histamine was released into the extract but no 'SRS' could be detected. If, however, sensitized tissues were treated with antigen before extraction with alcohol, both histamine and 'SRS' could be detected in the extract. Freezing and thawing of the tissue released a large amount of total histamine but no 'SRS' was detectable either in the Tyrode solution in which the tissue was suspended or in the alcohol extract of the tissue. It may be mentioned here that in experiment 1 (Table III) the tissue was frozen in acetone+dry-ice and thawed at 0° to 3° C. In an experiment where the tissue was thawed at 37° C, a small amount of 'SRS' was found in the alcohol extract of the tissue following freezing and thawing. This discrepancy could possibly be explained by the assumption that disruption of cells during freezing and thawing released some enzyme which remained active at 37° C but was inactive at the low temperature.

Decylamine probably releases histamine by a direct lytic action (HÖGBERG and UVNÄS 1960). In concentrations of 25 and 100 μg/ml, it caused the release of 2.2 and 6.0 μg/g histamine from guinea pig lung pieces, but when ether

Table III. Showing absence of 'SRS' when histamine is released by physical destruction of the cells of guinea pig lung

Expt.	Alcohol extraction of tissue		Freezing and thawing of tissue		Alcohol extraction of tissue after freezing and thawing		Incubation fluid for anaphylactic reaction		Alcohol ext. of tissue after anaphylactic reaction	
	H	'SRS'	H	'SRS'	H	'SRS'	H	'SRS'	H	'SRS'
1	5.1	0	4.0	0	0.81	0	—	—	—	—
2	5.5	0	—	—	—	—	2.3	256	4.2	136

H = histamine μg/g tissue. 'SRS': expressed in units/g tissue.

extract of the incubation fluid was tested on guinea pig ileum no 'SRS' could be detected. These observations indicate that when histamine is released by some physical process of cell disruption, it is not attended with the appearance of 'SRS'. 'SRS' can be extracted from the tissue only after incubation with antigen which, in the light of the investigations reported here, activates an enzymatic process.

Discussion

Recent observations showing that histamine release in anaphylaxis is influenced by temperature, pH, Ca^{++} ions and enzyme inhibitors, point to the activation of an enzyme in the process (MONGAR and SCHILD 1957, 1958, CHAKRAVARTY 1960 a, HÖGBERG and UVNÄS 1960).

The investigations reported here show close parallelism between the occurrence of histamine and that of 'SRS' in anaphylactic reaction in guinea pig lung. Factors mentioned above — pH, temperature, Ca^{++} ions, anoxia, enzyme inhibitors — cause similar changes in the occurrence of the two smooth-muscle stimulating principles. In our experiments no 'SRS' was detected without concomitant release of histamine. These observations strongly suggest that either the same or similar enzyme mechanisms are instrumental in the appearance of histamine and 'SRS'.

Since mast cells disrupt during anaphylactic reaction in guinea pig tissues (MOTA 1958, BORÉUS and CHAKRAVARTY 1960) and histamine is released from them, the question arises as to whether or not they are the source of 'SRS' as well. The answer is not necessarily affirmative, since in antigen-antibody reactions various tissue cells may release or form biologically active substances. In this connection it is of interest to note the observations of CHAKRAVARTY, HÖGBERG and UVNÄS (1959) concerning the appearance of histamine and 'SRS' from perfused cat paw upon addition of compound 48/80 to the perfusion fluid. As far as we know, compound 48/80 in the doses used in these experiments acts

selectively upon mast cells and releases histamine. CHAKRAVARTY *et al.* found that 'SRS' and histamine not only occurred together but showed parallel variations similar to those observed in anaphylactic reaction in guinea-pig lung tissue, as reported in this paper. Bearing in mind the selective action of compound 48/80 on cat-tissue mast cells, it is plausible to assume that the 'SRS' obtained from cat paw either originates directly from the latter's mast cells or is secondary to their disruption. The same might well be true of the 'SRS' appearing in guinea-pig lung tissue when its mast cells disrupt under the influence of antigen-antibody reaction, the more so because a close chemical and biological relationship probably exists between the 'slow reacting substances' in the two species (CHAKRAVARTY 1959). Recently, UVNÄS and THON (1959) found a slow reacting substance to occur together with histamine in a mast cell suspension, when the mast cells were exposed to compound 48/80.

Histamine and 'SRS' appear concomitantly upon addition of antigen to sensitized guinea-pig lung. If, however, histamine is extracted from minced tissue, using alcohol or other mild extraction procedures — before or after freezing and thawing of the tissue — no 'SRS' is obtained. Yet extraction of the tissue after addition of antigen, yields both histamine and 'SRS'. The latter is evidently formed in the tissue during anaphylactic reaction, whereas histamine is simply released from its existing source in the mast cells. This is corroborated by our observations in experiments with compound 48/80 on cat tissue (CHAKRAVARTY *et al.* 1959).

The difference between the behaviour of histamine and that of 'SRS' could be explained by the theory of HÖGBERG and UVNÄS (1957), according to which the degranulation of mast cells caused by synthetic polymeric histamine liberator (at least in cat and rat), and by antigen, is due to activation of a lytic enzyme attacking the phospholipids of the mast-cell membrane. Fatty acids and other acid lipid substances with smooth-muscle stimulating properties are known to occur in the body; they include prostaglandin (EULER 1936, ELIASSON 1959, BERGSTRÖM *et al.* 1959), irin (AMBACHE 1957), G-acid (GABR 1956), etc. If the theory of HÖGBERG and UVNÄS is correct, lipid split products could be formed. 'SRS' coinciding with histamine release could be such an acid lipid, smooth-muscle stimulating principle which occurs on activation of the lytic enzyme.

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Tissue Mast Cells, Histamine and 'Slow Reacting Substance' in Anaphylactic Reaction in Guinea Pig

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Abstract

BORÉUS, L. O. and N. CHAKRAVARTY. *Tissue mast cells, histamine and 'Slow Reacting Substance' in anaphylactic reaction in guinea pig.* Acta physiol. scand. 1960. *48.* 315—322. — Anaphylactic reaction was produced *in vitro* in isolated guinea-pig tissue. Histamine and 'slow reacting substance' ('SRS') in different tissues occurred in amounts proportional to the mast-cell population. The occurrence of these substances was attended with a reduction of the number of tissue mast cells. When the appearance of histamine and 'SRS' was blocked by enzyme inhibitors, there was no decrease in mast cells. It is suggested that the tissue mast cells may be the common source of both histamine and 'SRS' in anaphylactic reaction.

Since the observation that histamine is located in the mast cells (RILEY and WEST 1953), interest has been focussed on changes in these cells in anaphylactic reaction. It has been demonstrated that the mast-cells of sensitized guinea pigs disintegrate and disappear following injection of antigen (MOTA and VUGMAN 1956); and that, moreover, the mast-cell disappearance increases with rising concentrations of antigen both *in vitro* (MOTA 1958) and *in vivo* (BORÉUS, to be published).

It was recently reported from this laboratory that not only histamine but a lipid-soluble smooth-muscle stimulating principle — probably the same as the slow reacting substance ('SRS') of KELLAWAY and TRETHEWIE (1940) — occur in anaphylactic reaction, and that a marked quantitative correlation exists between the two substances under various experimental conditions (CHAKRAVARTY 1960 b, CHAKRAVARTY and UVNÄS 1960). The observations suggest that histamine and 'SRS' appear in anaphylactic reaction by virtue of the same or similar mechanisms and possibly stem from a common source. In the present investigation the occurrence of histamine and 'SRS' in anaphylaxis has been correlated with the disappearance of mast cells from sensitized tissues.

Material and Methods

Guinea pigs, male or female, 250 to 350 g, were sensitized by two injections of egg albumin subcutaneously and intraperitoneally within the space of a week. Anaphylactic reaction was produced *in vitro* in isolated tissues 4 to 12 weeks after the first injection. The tissues were cut into pieces, then washed and incubated in Tyrode solution with antigen. Details of this method have already been described (CHAKRAVARTY 1960 a). To ensure maximal uniformity of the initial histamine content and mast-cell population of the tissue pieces, only one animal was used for each experiment. Four to six samples, each weighing 0.3 to 0.4 g (wet weight) could be obtained from the lungs of each animal, thus permitting evaluation of the effect of inhibitors or other experimental procedures. Following the anaphylactic reaction *in vitro*, the incubation fluid was collected for histamine and 'SRS' assay, and the tissue was fixed as described below for mast-cell counts. Control samples were incubated in Tyrode solution without antigen for corresponding periods before fixation, and the incubation fluid was similarly tested. Spontaneous release of histamine in control samples was either very low or nil. When detectable, the quantity was deducted in order to obtain the anaphylactic histamine release. No spontaneous 'SRS' release was detected.

When an inhibitor of anaphylactic reaction was used, the tissues were incubated with it for 15 to 20 min before addition of antigen. In the case of 2:4-dinitrofluorobenzene and acetic anhydride the tissue was washed twice in a relatively large volume of Tyrode solution following treatment with the inhibitors, and was then incubated in Tyrode solution with antigen.

For mast-cell counts the specimens were fixed in 4 per cent lead subacetate in 50 per cent ethyl alcohol with 1 per cent acetic acid. Frozen sections, 100 μ thick for lungs and 30 μ for the other tissues, were stained in 0.5 per cent toluidine blue in water. In the experiments with lung tissue, six specimens from each sample were sectioned, and for each specimen 10 visual fields, chosen at random, were counted at a magnification of 360×. In this way the total mast cells in 60 fields were counted for each sample. In the case of other tissues, the area of each section was estimated with an ocular micrometer and the total mast cells in the entire section were counted. As a rule, two specimens from each sample were sectioned and two sections of each specimen subjected to counts. The results were calculated as the number of mast cells per unit area (0.256 mm^2). To obviate subjective error in counting, the investigator was left unaware of the experimental procedure for the individual samples.

Histamine was assayed biologically on atropinized guinea-pig ileum and the values are expressed as histamine base. 'SRS' was assayed in arbitrary units on guinea-pig

ileum in the presence of atropine and an antihistaminic (mepyramine) against a standard prepared in this laboratory as described previously (CHAKRAVARTY 1959). When an inhibitor, *e. g.* iodoacetate, was present in the test sample, it was added in proportional amounts to the standard histamine and 'SRS'. When alcohol was used as an inhibitor, the incubation fluid was freeze-dried and redissolved in water before testing on guinea-pig ileum.

Tissue histamine was extracted by a slight modification of the method of FELDBERG and TALESNIK (1953).

The experiments were duplicate (unless otherwise stated), and the mean values are presented.

Results

When sensitized guinea-pig tissue is incubated with antigen, the mast cells show loss of metachromatic stainability, fragmentation of the cell body and decrease in the number of detectable cells. The most reliable of the criteria which may be used for determining the effect of antigen on mast cells, is the number of detectable cells, other changes being variable and difficult to evaluate.

Table I. Mast-cell count and appearance of histamine and 'SRS' in anaphylactic reaction of guinea-pig lung. Incubation time with antigen (1 mg/ml) 10 minutes

Mean values and standard errors from 7 experiments

Mast cells per 0.256 mm²			Histamine μg/g	'SRS' units/g
Control (without antigen)	With antigen	% disappearance		
6.14 ± 1.10	2.56 ± 0.42	57.4 ± 6.0	3.91 ± 0.82	311 ± 86
	P = 0.01			

Table I shows the mast-cell count in sensitized guinea-pig lung pieces incubated with or without antigen; the difference is expressed as per cent disappearance in anaphylactic reaction. As shown in the table, about one-half of the mast cells disappeared from the tissue after 10 min incubation. During the same period considerable amounts of histamine and 'SRS' appeared in the incubation fluid.

Time Course

The time course of mast-cell disappearance and the occurrence of histamine and 'SRS' for the first 8 min is shown in Fig. 1. Following the addition of antigen there was progressive mast-cell depletion, and increasing amounts of histamine and 'SRS' occurred in the incubation fluid. In the initial phase, 'SRS' appeared at a slower rate than histamine, as shown previously (CHAKRAVARTY 1960 b).

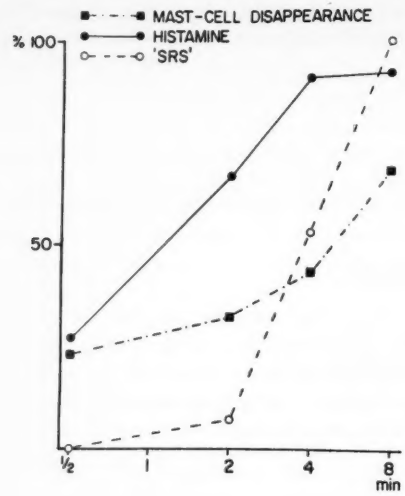


Fig. 1. Time course of histamine and 'SRS' appearance and of mast-cell disappearance from sensitized lung pieces following the addition of antigen (1 mg/ml). Histamine and 'SRS' expressed as per cent of their maximum values; mast-cell counts expressed as per cent disappearance.

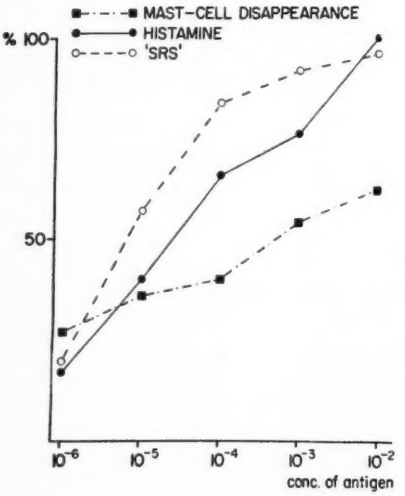
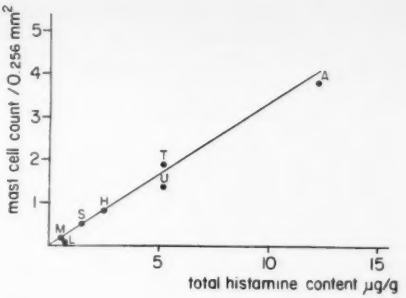


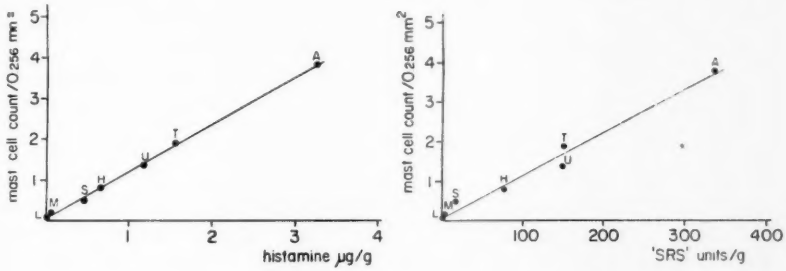
Fig. 2. Influence of concentration of antigen (w/v) on the appearance of histamine and 'SRS' and on the disappearance of mast cells from sensitized lung pieces. Histamine and 'SRS' expressed as per cent of their maximum values; mast-cell counts expressed as per cent disappearance. Incubation time with antigen 10 minutes.

Concentration of Antigen

With different concentrations of antigen the mast-cell loss increased progressively from 27 to 63 per cent when the concentrations rose from 10^{-6} to 10^{-2} (w/v), as shown in Fig. 2. Concomitantly, increasing amounts of histamine and 'SRS' appeared in the incubation fluid.

Fig. 3. Correlation of mast-cell count with histamine content in different tissues of guinea pig.
A = aorta,
T = trachea,
U = uterus,
S = abdominal skin,
H = heart,
L = liver,
M = striated muscle.





(a) (b)

Fig. 4. Correlation between mast-cell count and anaphylactic appearance of histamine (a) and of 'SRS' (b) in different guinea-pig tissues. Incubation time with antigen (1 mg/ml) 10 minutes. Abbreviations as in Fig. 3.

Mast Cells, Histamine and 'SRS' in Different Tissues

As shown in Fig. 3, a marked correlation was found between the mast-cell count and the total histamine content over a wide range in seven different tissues of guinea pig. This supports the view that most of the tissue histamine is located in the mast cells.

Fig. 4 shows a close correlation between the mast-cell count in different tissues (before incubation with antigen) and the occurrence of histamine and 'SRS' in anaphylactic reaction.

Tissues with a relatively high mast-cell count, *viz.* aorta, trachea and uterus, were also investigated for mast-cell depletion in anaphylactic reaction. As in the case of lung pieces, incubation with antigen caused a depletion of mast cells with concomitant appearance of histamine and 'SRS' (see Table II).

The intestine (jejunum) is exceptional in combining a high mast-cell count and a high histamine content with very poor release. In three experiments the histamine release was 0 to 3 per cent of a total histamine content ranging from 10.9 to 14.7 µg/g, and no 'SRS' whatsoever was detected. The lack of an appreciable release is reflected in the absence of mast-cell disappearance (see Fig. 5).

Table II. Mast-cell depletion and appearance of histamine and 'SRS' from guinea-pig aorta, trachea and uterus in anaphylactic reaction. Incubation time with antigen (1 mg/ml) 10 minutes

Mean values from 3 experiments

	Mast-cell depletion per 0.256 mm²	Histamine µg/g	'SRS' units/g
Aorta	1.84	3.8	213
Trachea	0.98	1.6	75
Uterus.....	0.92	1.8	102

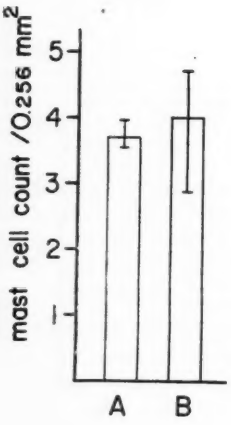


Fig. 5. Mast-cell count in sensitized guinea-pig jejunum incubated without (A) and with (B) antigen. Incubation time with antigen (1 mg/ml) 10 minutes. Mean values and range from three experiments.

Effect of Inhibitors

The correlation between mast cells and the appearance of histamine and 'SRS' is also demonstrated by the use of inhibitors. Four compounds were used as inhibitors, viz. ethyl alcohol, the sulphydryl-blocking agent iodoacetate, and 2:4-dinitrofluorobenzene and acetic anhydride, both of which react with the amino groups of proteins. These compounds have been shown to block the anaphylactic appearance of histamine and 'SRS' in guinea pig (CHAKRAVARTY and UVNÄS 1960) and the disruption of mast cells in rats (HÖGBERG and UVNÄS 1960). As shown in Table III, virtually no histamine or 'SRS' could

Table III. Effect of inhibitors on histamine and 'SRS' appearance and on mast-cell depletion in sensitized guinea-pig lung. Incubation time with antigen (1 mg/ml) 10 minutes

Inhibitor		Mast cells (per 0.256 mm²)	Histamine µg/g	'SRS' units/g
Iodoacetate 10^{-3} M	Control	4.9	0.08	0
	Antigen	2.4	7.0	490
	Inhibitor + antigen	5.7	0.62	34
Dinitrofluorobenzene 10^{-3} M	Control	4.3	0	0
	Antigen	1.8	2.3	61
	Inhibitor + antigen	4.4	0.11	0
Acetic anhydride 10^{-2} M	Control	4.3	0	0
	Antigen	1.8	2.3	61
	Inhibitor + antigen	4.1	0.07	0
Ethyl alcohol 10^{-2} v/v	Control	6.7	0.17	0
	Antigen	2.6	4.8	323
	Inhibitor + antigen	5.3	2.1	114

be detected in the incubation fluid following the use of enzyme inhibitors, and no significant change was observed in the mast cell count; with ethyl alcohol, which was less inhibitory in effect, the results were similar.

Discussion

Riley and West (1953) suggested, on the basis of the correlation between histamine content and mast-cell population of tissues, that histamine is located in the mast cells. This view was supported by the high histamine content of isolated mast cells (Benditt, Arase and Roeper 1956) and mastocytomas (Cass *et al.* 1954). It was subsequently shown, by the use of C^{14}-labelled L-histidine, that mast cells readily form and bind histamine from its precursor (Schayer 1956; Lindell, Rorsman and Westling 1959).

Antigen-antibody reaction triggers anaphylactic shock and releases histamine from its site of binding in the mast cells. Another smooth-muscle stimulating principle occurs concomitantly with the released histamine (Chakravarty and Uvnäs 1960, Chakravarty 1960 b).

In the present experiments it has been demonstrated that the occurrence of histamine and 'SRS' from the tissues is attended with mast-cell depletion. With increasing concentrations of antigen, the histamine and 'SRS' values of the incubation fluid increased more or less proportionally with progressive diminution of the mast-cell counts of the tissue. The time curve shows that when increasing amounts of histamine and 'SRS' appear in the incubation fluid, there is continued loss of mast cells from the tissue. The mast cells, it would seem, react almost immediately on contact with the antigen, and the

delayed depletion is due, presumably, to the time required for diffusion of the antigen into the deeper parts of the tissue. This is corroborated by the rapid disappearance of mast cells when the diffusion time is minimized; intra-arterial injection of antigen resulted in maximal depletion within one minute in guinea-pig nasal mucosa (BORÉUS, to be published).

Intestine differed from other tissues in having a high mast-cell count with no depletion in anaphylactic reaction. This is reflected in the absence of the appearance of any appreciable amount of histamine and 'SRS', although the extractable histamine content is high. Apparently, the intestinal mast cells are more resistant to disintegration when incubated with antigen. The reason for this is not clear from the present investigation.

The observations in this paper show a parallelism between the appearance of histamine and 'SRS' and the disappearance of mast cells. This could be explained by assuming that both substances stem from a common source, *viz.* the mast cell.

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Acta physiol. scand. 1960. 48. 323—328

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The Effect of Ethyl Alcohol on the Secretion from the Adrenal Medulla of the Cat

By

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Abstract

PERMAN, E. S. *The effect of ethyl alcohol on the secretion from the adrenal medulla of the cat.* Acta physiol. scand. 1960. *48.* 323—328. — Recent studies on the urinary excretion of adrenaline and noradrenaline have furnished strong indirect evidence that ethyl alcohol administration increases the secretion from the adrenal medulla in man and dogs. In the present work the amounts of adrenaline and noradrenaline in the blood from one adrenal were measured after alcohol infusions (0.30—1.0 g/kg) in Nembutal-anaesthetized cats. The catecholamines were determined by bioassay technique. With i. v. doses of alcohol above 0.60 g/kg, the output of both amines generally increased. Some ways in which alcohol could influence the adrenal medulla are discussed.

The relationship between ethyl alcohol and various organs with an autonomic nerve supply has recently received some attention. Apparently only KLINGMAN and co-workers (KLINGMAN and BANE 1955, KLINGMAN and GOODALL 1957, KLINGMAN and HAAG 1958, KLINGMAN, HAAG and BANE 1958) have studied the effects of alcohol on the adrenal medulla in animals. In dogs with severe alcohol intoxication after doses of 6.4—12 g/kg the adrenal catecholamine content was reduced and the urinary excretion of adrenaline and noradrenaline increased. Plasma glucose, potassium and other parameters also indicated a strong release of adrenaline and noradrenaline. Adrenalectomy indicated that the adrenal medulla was chiefly responsible for the increased catecholamine release. Hexamethonium treatment suggested that the alcohol effect on the adrenal was mediated via nervous pathways. In other reports (HAAG, KLINGMAN and BANE 1957, KLINGMAN, BANE and HAAG 1959) strong

Table I. Secretion of adrenaline and noradrenaline from the adrenal medulla into the left suprarenal vein after i.v. infusion of ethyl alcohol as compared to the "resting" secretion

Exp. no.	Resting secretion ng/kg/min.		Dose of ethyl alcohol g/kg	Secretion after i.v. alcohol infusion ng/kg/min.		Time in min. between end of alcohol administration and sample where highest secretion was found
	adrenaline	noradrenaline		adrenaline	noradrenaline	
1	< 2	18	0.30	3	45	10
2	< 1	14	0.50	4	15	15
3a	< 2	26	0.48	< 2	30	5
3b	< 2	25	0.64	20	108	15
4	< 2	19	0.60	25	79	20
5	< 1	18	0.65	35	147	55
6	< 3	30	1.0	400	53	5

indirect evidence that lower alcohol doses 3.2 g/kg also exert similar actions was presented. Blood pressure and respiration data were not reported.

PERMAN (1958) found that in human subjects who had received moderate amounts of alcohol, the urinary adrenaline excretion was elevated. The noradrenaline excretion remained mainly unchanged. Increased release from the adrenal medulla was thought to be responsible for the effect. Similar results have been reported by ABELIN, HERREN and BERLI (1958). In their experiments the urinary noradrenaline excretion was also increased after alcohol administration.

The aim of the present investigation was to study the effect of moderate amounts of alcohol directly on the release of adrenaline and noradrenaline from the adrenal medulla in the anaesthetized cat.

Material and Methods

Six cats received alcohol infusions, and in addition three control experiments were performed. Animals weighing 3.8 to 5.0 kg were used. They were anaesthetized with Nembutal® 35 mg per kg body weight intraperitoneally and heparinized. A tracheal cannula was inserted and the femoral vein was cannulated. The blood pressure was recorded from one of the carotid arteries. The left suprarenal vein and lateral branches of the left lumbar vein were ligated. The left adrenal venous blood was then shunted through a polyethylene tube from the left lumbar vein, through the abdominal wall into the cannula in the femoral vein, mainly as described by SCHAPIRO and STJÄRNE (1958). Because of this exteriorized system samples of the adrenal venous blood could be obtained without manipulations in the proximity of the adrenal gland. When samples were taken an equivalent volume of a Ringer or Dextran (Macrodex®) solution was infused into the animal in order to minimize changes in blood volume.

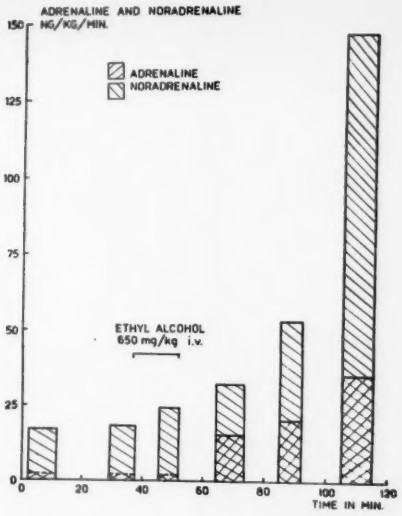

Fig. 1. Diagram of alcohol experiment no. 5. Secretion of adrenaline and noradrenaline from the left adrenal of a Nembutal-anaesthetized cat before and after i.v. infusion of ethyl alcohol 650 mg/kg.
(– – – – – represents upper limits of adrenaline release.)

The alcohol solution (16 per cent w/v in saline) was given in the femoral vein at a constant, slow rate.

Each sample of suprarenal venous blood had a volume of 4—5 ml. After collection the sample was immediately centrifuged, the hematocrit estimated, and the plasma pipetted off. Adrenaline and noradrenaline in the plasma were determined by bioassay (cat blood pressure and hen rectal caecum) according to EULER (1949). When bioassay was not performed immediately, the samples were stored at — 30° C. All figures are given as ng (1 ng (nanogram) = 1 mμg) per kg per min. in terms of the hydrochlorides of the amines.

The experiments were begun not earlier than half an hour after the end of the operative procedure. First, samples of the "resting" secretion were taken. Alcohol was then given and further samples collected at intervals. The experiments usually lasted $1^1/_2$ hours. In few experiments the adrenal vein blood was collected continuously. These experiments had a shorter duration.

Results

Table I shows the results of the alcohol experiments. In the resting secretion adrenaline could not be clearly demonstrated with the bioassay systems used and the noradrenaline values were low. After administration of alcohol in doses above 0.60 g/kg the output of both amines generally increased, sometimes after a latency period. The alcohol-induced release tended to come gradually and could usually be followed in several samples. In experiment no. 3 an increase was obtained only after an additional dose of alcohol was given. The relative amounts of adrenaline and noradrenaline in the secretion after alcohol administration varied considerably in different experiments.

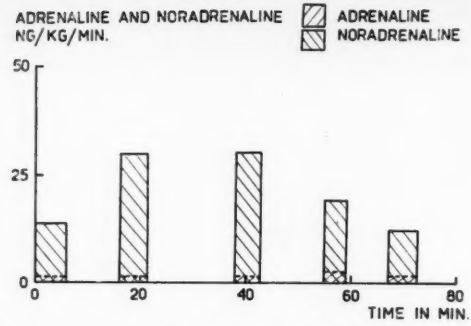

Fig. 2. Diagram of one control experiment. Secretion of adrenaline and noradrenaline from the left adrenal of a Nembutal-anaesthetized cat during a 70 min period. (– – – – – represents upper limits of adrenaline release.)

Only minor fluctuations in the blood pressure were seen during the experiments. Alcohol, as a rule, had a slight pressor action and an increased pulse frequency was noted after the alcohol infusions.

In experiment no. 6 a larger alcohol dose (1.0 g/kg) produced a very strong increase in the adrenaline release from 3 ng/kg/min to 400 ng/kg/min after which the adrenaline values gradually returned to the "resting" level. The noradrenaline release was only slightly increased at the same time. A moderate pressure fall and respiratory depression were seen after the alcohol infusion in this experiment.

In the three control experiments which had approximately the same duration as the alcohol experiments, corresponding amounts of adrenal vein blood were withdrawn for bioassay. At the beginning of these experiments the adrenaline and noradrenaline values showed good agreement with the values for the "resting" secretion in the alcohol experiments. The noradrenaline secretion fluctuated somewhat during the experiments but no consistent change in the catecholamine output was observed. Fig. 2 shows a diagram of a control experiment. In another control experiment a saline infusion was given. This decreased the output of catecholamines moderately during a period of 20 min.

A reduction (5—10 %) in the hematocrit value occurred gradually during some of the experiments, probably due to the removal of blood for catecholamine determination. This reduction did apparently not influence the release of adrenaline and noradrenaline.

Discussion

The amounts of adrenaline and noradrenaline in the "resting" secretion compare fairly well with values obtained by other workers. The adrenaline

secretion is somewhat low and it can be assumed that the "stress" induced by the anaesthesia and the preparation of the animal has been moderate.

The amounts of catecholamines in the suprarenal venous blood increased when alcohol doses of 0.60 g/kg and above were infused, whereas in experiments with lower doses (0.30—0.48 g/kg) and in control experiments only minor changes occurred. There is little reason to believe that the changes are of unspecific nature, for instance secondary to activation of the baroceptor mechanisms or to asphyxia. The general condition of the animals as judged from arterial pressure and respiration, was largely unaffected by the alcohol doses used. The slight pressor action often noted after alcohol administration should, if anything, counteract a release of catecholamines from the adrenal medulla. In experiment no. 6 where 1.0 g/kg alcohol was given, blood pressure and ventilation decreased, but this would hardly explain the strong selective adrenaline output that occurred in this experiment.

In experiments of this type the general anaesthesia and other factors will naturally render analysis of the mechanism for alcohol action difficult but some assumptions may be made. The proportions of adrenaline and noradrenaline released by alcohol varied considerably. These variations in the relative amounts suggest that the effect is mediated via nervous pathways. It is known (Brücke, Kaindl and Mayer 1952, Folkow and Euler 1954) that the adrenal medullary secretion, induced by electrical stimulation in different parts of the hypothalamus, shows marked variations in the relative amounts of adrenaline and noradrenaline. Dunér (1953, 1954) has shown that blood sugar changes more or less selectively influence the adrenaline secretion possibly through a mechanism located in the hypothalamic area. There is no evidence at present for a direct effect of ethyl alcohol on the chromaffin cells. A more detailed study of the mechanism is in progress.

Alcohol is assumed to affect different levels in the central nervous system progressively, beginning in the highest level, the cortex. Results of electrical stimulation of the orbital cortex suggest that this area normally inhibits the secretion from the adrenal medulla (Euler and Folkow 1958). Alcohol, acting on the cortex, might relieve hypothalamic centers from this inhibiting influence thus causing increased adrenal medullary secretion. Another possibility is that alcohol acts on the previously mentioned mechanism concerned with the glycemic homeostasis.

The significance of the activation of the adrenal medulla caused by the alcohol doses used in these experiments is as yet not clear. In the reports of Klingman and co-workers high alcohol doses causing lethal or sublethal intoxication were used. These doses, which among other things produced circulatory and respiratory reactions, would surely "stress" the experiment animals. A response from the adrenal medulla can therefore be expected. However, these investigators (Haag, Klingman and Bane 1957, Klingman, Bane and Haag 1959) have, as mentioned earlier, presented strong indirect evidence

that alcohol releases adrenaline and noradrenaline from the dog adrenal also in lower doses (3.2 g/kg).

In the present investigation alcohol in a low dose-range was given and the general condition of the animals remained mainly unaffected. Increased output of adrenaline and noradrenaline from the adrenal was still found. It appears likely that alcohol brings about this release in a way different from unspecific "stress". ABELIN, HERREN and BERLI (1958) have suggested that the effect has metabolic significance. In their opinion, the release of catecholamines in the body after alcohol administration forms a kind of "defence" mechanism whereby the body accelerates the disappearance of alcohol by increasing its oxidation.

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Acta physiol. scand. 1960. 48. 329—351

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Interhippocampal Impulses

IV. A Correlation of some Functional and Structural Properties of the Interhippocampal Fibres in Cat, Rabbit and Rat

By

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Abstract

ANDERSEN, P. *Interhippocampal Impulses. IV. A correlation of some functional and structural properties of the interhippocampal fibres in cat, rabbit and rat.* Acta physiol. scand. 1960. *48.* 329—351. — In a previous study (ANDERSEN 1959, 1960 b) the interhippocampal fibre activity was found to be represented by an initial diphasic spike of the response of the field CA3 to contralateral symmetrical stimulation. Since all the interhippocampal fibres are thin (around 1 μ) and myelinated, it was felt that this tract might serve as a suitable structure for a study of the properties of thin fibres within the central nervous system. A correlation has been made between various functional and structural properties of the interhippocampal fibres. The results obtained in cat, rabbit and rat have been compared.

Introduction

In a previous paper the commissural response of the hippocampal field CA3 was described as consisting of two main parts, a diphasic spike and a subsequent negative wave (ANDERSEN 1959, 1960 b). Evidence was presented to indicate that the diphasic spike was due to the activity of interhippocampal fibres in the fimbria while the negative wave was considered as a postsynaptic potential elicited in the CA3 neurons.

The present article is concerned with the behaviour of the diphasic spike only.

The only previous investigation of the physiological properties of the interhippocampal fibres is that of CRAGG and HAMLYN (1957). These authors found that the commissural hippocampal impulses in the rabbit travelled in the ventral part of the fimbria, and near the base of this structure. The conduction velocity was calculated to 12.5 m/sec.

The physiological properties of some other central fibre tracts have been investigated: the pyramidal tract fibres (ADRIAN and MORUZZI 1939, LLOYD 1941, WOOLSEY and CHANG 1947, BISHOP, JEREMY and LANCE 1953b, and LANCE 1954), the optic nerve and tract (BISHOP, JEREMY and LANCE 1953 a, BISHOP 1953, BISHOP and CLARE 1955, CHANG 1956, and LENNOX 1958), and cortical afferent fibres from the splanchnic nerve (AMASSIAN 1951). However, these tracts contain, in addition to the thinner axons, a considerable number of fibres with diameters greater than 3 μ. As reported by several authors, the fornix and the ventral psalterium (ventral hippocampal commissure) are composed of very thin fibres only, the majority being less than 1 μ (CAJAL 1911, LORENTE DE NÓ 1934, DAITZ 1953, POWELL and COWAN 1955).

The aim of the present investigation was to make a comparison between some functional and structural properties of the interhippocampal fibres, and in addition, to correlate these properties with those of fibres in other tracts within the central nervous system and with peripheral nerve fibres. Special attention was paid to the existence of species differences concerning the properties of the hippocampal fibres.

Material and Methods

Physiological studies. — The results are based on experiments in 18 cats (0.17—5.6 kg), 11 rabbits (2.0—3.4 kg), and 9 rats (0.25—0.40 kg). The operational procedure and the stimulation and recording techniques have been described elsewhere (ANDERSEN 1959, 1960 a). Urethane-chloralose anesthesia and conventional evoked potential technique with small electrodes were employed.

The conduction velocity was measured from a graph in which the distances between the stimulating electrode and various recording points along the fimbrio-hippocampal border were plotted against the respective latencies of the diphasic spike. The conduction velocity is given by the slope of the connecting line (HURSH 1939). The recording points were located in the strip shaped area of the fimbria where the highest amplitudes of the diphasic spike were noted (ANDERSEN 1959). These points were marked by applying india ink to the electrode tip. The conduction distances were measured on the preparation *in situ* by a pair of compasses and controlled in the same manner after removal of the brain at the end of the experiment. The mean value of 5 measurements was used. When the electrode was located near the temporal end of the fimbria, where the fibres have a fairly curved course, the distance was measured by placing a thin thread (BISHOP *et al.* 1953 a) along the fimbria. In some early experiments the measurement of the distances was controlled by photography with a millimeter paper strip along the fimbria. As the values obtained in this manner

showed deviations of less than 5 per cent from those obtained with the pair of compasses, the latter method was later used as a routine. The latency was measured to the point where the rising phase of the diphasic spike intersected the baseline (BISHOP *et al.* 1953 a), or, when the stimulus artifact distorted the latter, to the steepest part of the rising phase. No significant difference in the conduction velocity was found with the two methods. No satisfactory monophasic fibre potential was obtained.

The environmental temperature of the interhippocampal fibres was controlled by a paraffin pool, refilled completely every 2 minutes with liquid paraffin at 38° C. The temperature was measured by means of two thermocouples. One was placed on the fimbrial surface between the two fixed recording electrodes, and the second in the paraffin pool. The temperature of the operating room was kept at about 25° C. Under these conditions the temperature of the paraffin pool and of the fimbrial surface remained remarkably constant at approximately 34° C (33.2—34.5° C). Following complete refilling of the pool with paraffin at 38° C the temperature of the pool decreased to the usual one of about 34° C in about one minute. Temperature variations were produced locally by constant irrigation of the pool with paraffin at either 8° C or 50° C. General cooling was achieved by immersing the animal to the neck in ice-water after shaving and immobilization with decamethonium bromide (cat 0.1 mg/kg, rabbit 0.25 mg/kg). Under local cooling or heating the inlet site of the paraffin was at least 2 cm removed from either fimbria. For the determination of the variations in the conduction velocity induced by temperature changes, two fixed monopolar recording electrodes, about 5 mm apart, were used.

The effect of anoxia on the conduction velocity was similarly studied by two simultaneous monopolar recordings. First, the animals received an intravenous injection of decamethonium bromide (doses as given above), then the oxygen in the respiratory pump was replaced by nitrogen. Care was taken to prevent overbreathing.

Histological studies. — In this part of the investigation 7 cats (2.3—3.3 kg), 8 rabbits (2.0—3.2 kg) and 4 rats (0.22—0.45 kg) were used.

In order to measure the diameter of the fibres in the ventral psalterium, midline sagittal sections of the brain were employed. Good results were obtained in 2—3 μ thick sections from 0.5—1 mm thick blocks of tissue fixed in a 1 per cent solution of osmium tetroxide, buffered to pH 7.4 by a veronal-acetate buffer, and subsequently treated according to the method of SCHULTZE (1906). Valuable results were also obtained by phase contrast microscopy. Unfixed tissue was cut in sections of 10 μ on the freezing microtome. The sections were mounted in the animal's own blood plasma, and a cover glass was sealed by paraffin. Such preparations remained unchanged for 3—8 hours. After 24 hours, signs of myelin disintegration became apparent. — Photomicrographs were taken with a total magnification of 3,000 times. In addition, the fibre diameters were measured directly under the microscope, using an eye-piece micrometer. In each species 3,000 fibres were measured by both methods. These fibres were collected from 6 different areas within the ventral psalterium, each containing 500 fibres. Sections impregnated with silver according to the methods of BODIAN (1937) and NAUTA (1950) were found to be unreliable for measurement of fibre diameters. Information regarding the onset and degree of myelinization was gained from sections treated according to the methods of HÄGGQVIST (1936) and BAKER (1946). Two modifications of the WEIGERT technique, given by PAL (1886) and WOELCKE (1942), failed to show myelin within the ventral psalterium while the myelin around thicker fibres in the same sections was clearly stained.

In order to determine the total fibre content of the ventral psalterium, midline sagittal sections stained according to the method of NAUTA (1950) were used. The total area of the ventral psalterium was measured by projecting the sections, at a known magnification, on to a millimetre-squared graph paper. All fibres in 3 given sample

areas were counted in each of the three species studied. The areas counted measured 40 × 40 μ, and were located (i) in the ventralmost part, (ii) among the rostral bundles of fibres, and (iii) in the caudo-dorsal part of the ventral psalterium. By aid of a graticule, each field measured was subdivided into rectangles measuring 4.5 by 13.3 μ. The fibres were counted under an oil immersion lens. The total number of fibres was estimated by relating the density of the fibres to the cross-sectional area of the ventral psalterium. The fibres were counted in one animal from each species.

Results

A. Physiological observations

Form of the fibre potential. — Usually the activity of the interhippocampal fibres in the fimbria was recorded as a diphasic spike when the stimulating and recording electrodes were located symmetrically in the fields CA3 on the two sides. However, sometimes the negative wave, which usually followed, was lacking, and then the fibre activity appeared as a triphasic spike (Fig. 1A), the second, negative component being the dominant one. The form of the fibre potential was similar in cat, rabbit and rat. The negative component usually lasted 1—2 msec in rabbit and rat, while the duration in cat was 2—3 msec. The variations were partly due to difficulties in the measurement as the late part of the spike was often obscured by the following negative wave. These durations were recorded with the shortest possible distance between the stimulating and recording electrodes on the two sides.

Localization of the fibre potentials. — In agreement with the observations of CRAGG and HAMLYN (1957), the diphasic spike showing the greatest amplitude was recorded from the part of the fimbria lying adjacent to the hippocampus (Fig. 1 B). Near the midline, potentials with lower amplitudes, as compared with the responses obtained from more lateral electrode placements, were recorded. As the electrode was moved to the alveus, the fibre potentials became much smaller, suggesting that the commissural fibres in this region are more deeply located, or more spread out than is the case in the fimbria itself. Near the free, rostro-lateral margin of the fimbria, the fibre potentials were fairly small. This part of the fimbria is therefore presumably occupied by non-decussating axons.

In the experiment shown in Fig. 2 the distances between the stimulating electrode and the different recording points on each side of the midline were measured along the surface of the fimbria and the septal area. The interconnecting line in the graph shows an identical inclination (representing the conduction velocity) at the start and at the end, but with a discontinuity in the middle. This is apparently due to a longer conduction distance between the stimulating electrode and the contralateral recording points than that measured along the dorsal surface of the septum. Since it is most likely that the conduction velocity is the same all the way along the interhippocampal fibres, this finding suggests that these fibres bend ventrally to cross the midline

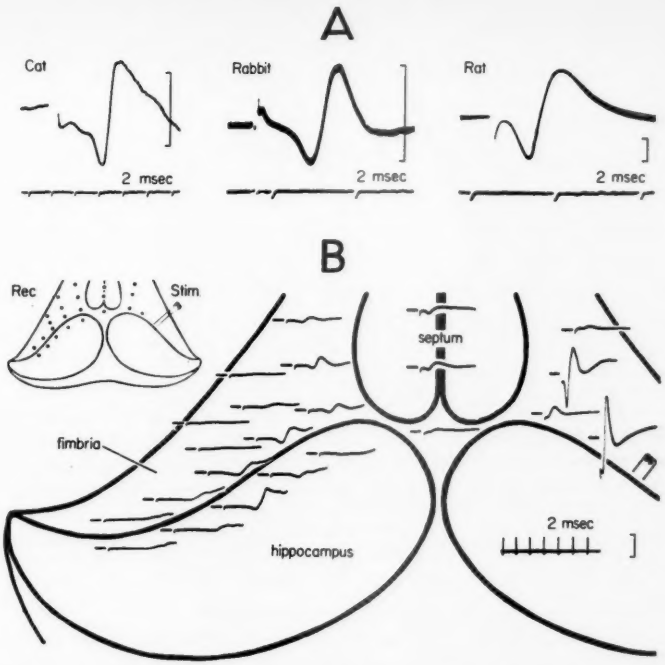

Fig. 1. *Form and localization of interhippocampal fibre potentials.* — A. Form and duration of the potentials in cat, rabbit and rat. — B. Localization of interhippocampal fibre potentials as recorded from various points on the ipsi- and contralateral hippocampus, fimbria and septum in response to stimulation at the fimbrio-hippocampal border in rabbit (see inset). Voltage calibration 0.5 mV.

In this and the following figures, the potentials are recorded monopolarly with negativity upwards.

in the depth. This was also found by histological methods (see below). The small amplitudes of the fibre potentials recorded from the midline as compared to those obtained 2—3 mm more laterally, is in agreement with this interpretation. From Fig. 2 it may be calculated that the increase in travelling distance because of such a ventral bend is 6 mm in cat, and from other experiments it was shown to be about 5 mm in the rabbit and approximately 2 mm in the rat.

Conduction velocity. — The graphs in Fig. 3 are obtained by plotting the distances in millimetres between a fixed stimulating electrode and a series of different recording points on the contralateral fimbria against the corresponding latencies of the diphasic spike. The conduction velocity of the interhippocampal fibres of the three species at 34° C may be read from the

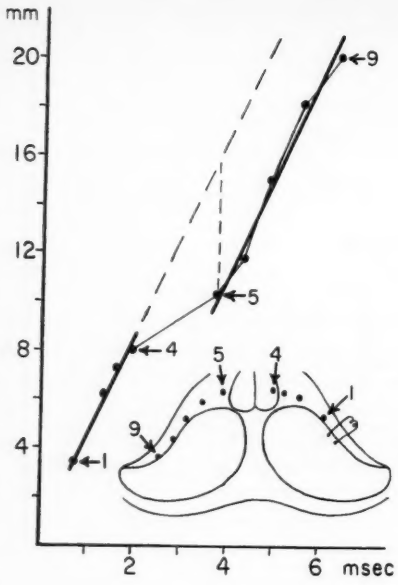

Fig. 2. Relation between the latency of the fibre spike and the distance, measured along the surface of septum and fimbria, to various electrode points along the ipsi- and contralateral fimbrio-hippocampal borders. Cat. Further explanation in text.

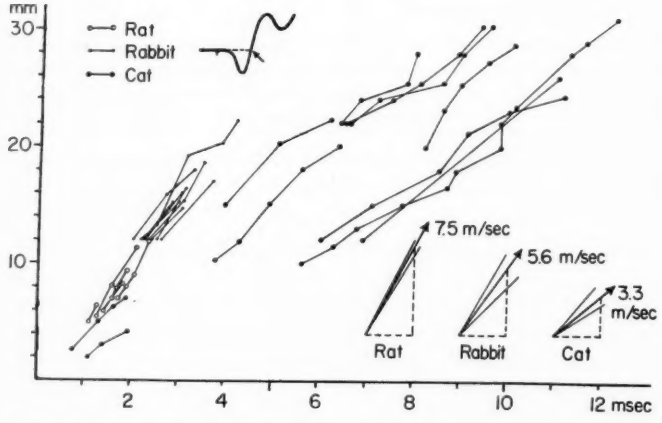

Fig. 3. *Graph showing conduction velocity of interhippocampal impulses in cat, rabbit and rat as measured at 34° C.* — Ordinate: Distance between the stimulating electrode and various electrode points along the contralateral fimbrio-hippocampal border. Abscissa: The corresponding latencies of the fibre spikes. — In the upper part of the figure the inset shows the point to which the latency was measured. In the lower right corner arrows are drawn indicating the mean values of the conduction velocities in cat, rabbit and rat, and the range of observations.

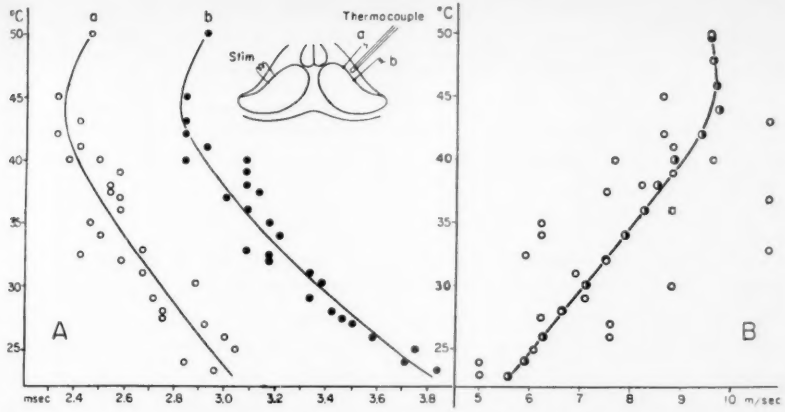

Fig. 4. *Relation between conduction velocity and temperature.* — A. Plotting of the latency of the diphasic fibre spike obtained by two fixed recording electrodes, *a* and *b*, against the varying local temperature. Each value mean of two observations. — B. Plotting of conduction velocity of interhippocampal impulses against the varying local temperature. Open circles: values calculated from each pair of observations. Half-filled circles: values obtained from the interconnecting lines drawn by hand in *A*. Distance *a-b* 4.4 mm. Rabbit.

slope of the interconnecting lines, or from Table I. This table also shows the number of experiments and animals used. The diagrams in the lower right corner of Fig. 3 show the mean values and the range of the conduction velocities measured in the three species.

Conduction velocity and temperature. — The results reported in this section are based upon 13 experiments in 7 rabbits and 2 cats. As expected, the conduction velocity decreased when the temperature of the paraffin pool was lowered from the usual 34° C. Fig. 4 demonstrates the results obtained in the rabbit experiment in which the highest conduction velocity was measured. In *A* the points represent the latencies of the diphasic spike as recorded by two fixed electrodes (*a* and *b*) at different temperatures. The conduction velocity was calculated from each pair of latency measurements (open circles in *B*), and from the mean value lines in *A* (half-filled circles in *B*). The latencies had their minima and the conduction velocity its maximum at about 44° C. The highest conduction velocity observed in rabbits was 9.7 m/sec, measured at 44° C (Fig. 4 B). Further increase of the temperature could result in irreversible damage to the fibres as judged from a smaller or absent diphasic spike on returning to physiological temperatures. The extent of the damage was dependent on the temperature and the duration of the exposure. No change was seen following irrigation with paraffin at 55° C for 20 seconds.

Table 1. Conduction velocity of interhippocampal fibres in adult cats, rabbits and rats (measured at 34° C)

Species	Number of experiments	Number of animals	Conduction velocity (m/sec)	
			Mean value	Range
Cat	9	7	3.3	2.5—4.8
Rabbit.....	11	8	5.6	4.0—7.6
Rat	5	5	7.5	6.1—7.8

The latencies were measured to the point where the rising limb of the diphasic spike intersected the baseline, or if the stimulus artifact distorted the latter, to the point of steepest slope of the spike.

Table II. Temperature coefficients (Q_{10}) for the conduction velocity of the interhippocampal fibres of rabbit at different temperatures

Temperature °C	Q_{10}	
	I	II
20—30	1.35	1.48
25—35	1.21	1.39
30—40	1.17	1.25
35—45		1.16
Maximum conduction velocity at 44° C (m/sec)	6.2	9.7

I—figures from an experiment in which the conduction velocity was about the average value

II — figures from the experiment in which the highest conduction velocity in rabbits were obtained.

The conduction velocity increased with higher temperature. However, the temperature coefficient (Q_{10}) decreased by the same procedure. The temperature coefficient of the conduction velocity of the interhippocampal fibres in the rabbit at different temperatures is given in Table II.

Conduction velocity and body weight. — The relationship between the conduction velocity of the interhippocampal fibres and the weight of the animal is presented in Fig. 5. Since in these experiments young animals had to be used, cats were chosen since kittens are far easier to operate upon than young rabbits and rats. Further, in the small brain of young animals, the kitten fimbria represents a larger target for the recording electrodes, and it is more accessible than the fimbria of rabbit or rat youngsters. Eight animals were used.

At body weights over 1.0 kg no clear relation between the conduction

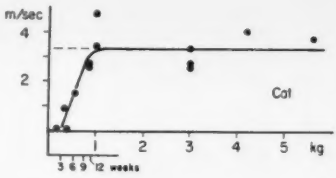

Fig. 5. *Relation between conduction velocity of interhippocampal impulses and body weight in kittens.* — Ordinate: Conduction velocity in m/sec at 34° C, obtained by the method shown in Fig. 3. Abscissa: Body weight in kg. Lower line indicates the approximate relation between the body weight and the age of kittens.

velocity and the weight of the animal was seen. This weight is usually reached when the kittens are 12—16 weeks old. In younger kittens the conduction velocity was lower. Commissurally evoked hippocampal potentials first appeared at a body weight of about 0.3—0.4 kg (about 2—3 weeks old). At this stage it is difficult to distinguish between the fimbria and the hippocampus, both being almost pellucid, suggesting that the fimbria contains little or no myelin. From this age on the conduction velocity increased until the adult value was obtained, at an age of about 12 weeks. As discussed below, this increase in the speed of propagation is apparently related to the myelinization of the fibres.

Impulse conduction and anoxia. — In Fig. 6 A the latencies of the diphasic spike, measured by two fixed recording electrodes with a separation of 4 mm, are plotted against the duration of an anoxic period of $4^1/_2$ min and a restitution period of 5 min. No clear effect on the latencies, and, therefore, not on the conduction velocity was noted in 5 similar experiments in cats.

However, if the anoxic period was lengthened, the latency of the diphasic spike increased with increasing duration of the anoxia (Fig. 6 B and C). The increase of the latency of the spike was paralleled by a decrease of the amplitude. Similar results with persistence of the diphasic spike during anoxic periods lasting for 20—35 min were obtained in 5 of 22 anoxia experiments (1 cat and 4 rabbits). In these five experiments the final abolition occurred rather suddenly.

Three other experiments (all in rabbits) differed somewhat from the described pattern. In one experiment the diphasic spike lasted for 40 min, in a second for 60 min and in a third it persisted for 100 min of anoxia. In the three latter experiments a more gradual decrease of the fibre potential was observed. The results obtained in these experiments are interpreted as due to oxygen uptake of the fibres directly from the dissolved air in the paraffin pool and, therefore, probably do not indicate an extreme resistance to anoxia of these fibres. Controls were made after each experiment to ensure that no leakage of air or oxygen had taken place into the respiratory system.

Refractory period. — The absolute refractory period of the interhippocampal fibres measured 1.2—1.3 msec in cats as well as in rabbits and rats (Fig. 7). The relative refractory period for all three species was 4—5 msec. The di-

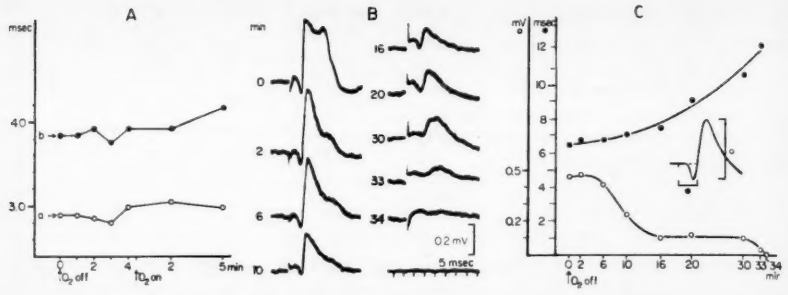

Fig. 6. *Effect of anoxia on impulse conduction.* — A. Latency of interhippocampal impulses recorded by two fixed recording electrodes *a* and *b* at the fimbrio-hippocampal border in a rabbit during an anoxic period of 4½ min, and a following restitution period of 5 min. No change in the latencies, and correspondingly no variation in the conduction velocity can be observed. — B. Interhippocampal fibre activity recorded at various duration of an anoxic period (cat). — C. Graph showing increase of conduction velocity and decrease of amplitude of the fibre potentials with increasing duration of the anoxia. Same experiment as in *B*.

phasic spike followed stimulation at frequencies up to 250/sec with a slight amplitude depression, in agreement with a relative refractory period of about 4 msec.

The absolute refractory period increased with lowered temperature, but the observations were too few to allow a calculation of the temperature coefficient for this process.

Period of latent addition. — By using subliminal stimuli and paired shocks, the maximal delay at which the diphasic spike could be elicited by the second shock was ordinarily 0.3 msec (7 experiments, rabbit). In 2 experiments a value as high as 0.65 msec was found. However, it is almost impossible to estimate the correct value of the period of latent addition of the interhippocampal fibres since the stimulation may easily spread to the CA3 neurons lying close to the fimbria. The long period of latent addition observed may, therefore, be a property of the CA3 cell bodies and not of the interhippocampal axons.

B. Anatomical observations

Topography of the ventral psalterium. — Fig. 8 shows a photomicrograph of a midline section through the brain of a rabbit, impregnated with silver according to the method of NAUTA and GYGAX (1954). The main part of the ventral psalterium is situated deeply at the rostral end of the third ventricle. When the horizontal levels of the hippocampus and the ventral psalterium are compared it is seen that in order to cross the midline the majority of the interhippocampal fibres must bend ventrally, some almost to the level of the anterior commissure.

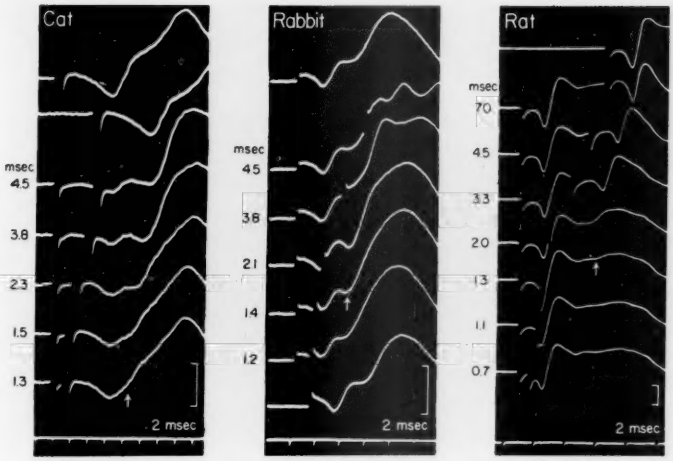

Fig. 7. *Excitability cycle of the diphasic fibre spike as tested by paired shocks in cat, rabbit and rat.* In cat, the first tracing shows the conditioning shock while the second shows the unconditioned test response. In rabbit these responses are shown in the first and last tracings respectively. In rat the first tracing shows the unconditioned test response. To the left of each paired shock tracing the delay is given in msec. The arrows indicate small traces of the test spike. Voltage calibration 1 mV.

The ventral psalterium is, in its caudal ventricular part, composed of fibres lying packed together in a fairly uniform pattern. In the rostral part, the fibres are gathered in bundles, between strands of fibres that course dorso-ventrally in a sagittal plane. In front of the ventral psalterium the fornix fibres curve downwards to the hypothalamic area.

Distribution of the interhippocampal fibres in the fimbria. — In one cat the ventral psalterium was severed near the midline. After a postoperative period of 18 days, horizontal sections of the hippocampi and the fimbriae were stained according to the method of MARCHI. Degenerating myelin particles were observed in the fimbria, but they were few and small compared with the degeneration in the internal capsule and in the optic tract which also were severed. The diameter of the degenerating particles in the fimbria ranged from 1.0—4.0 μ. They were found in all parts of it, but predominantly adjacent the hippocampus (CA3) and in the deep root of the alveus (penetrating into the hilus of the dentate fascia). Some degeneration was also observed in the dorsal alveus along its entire length towards the angular bundle. Following destruction of the one fimbria in two cats fibre degeneration in the regions described above was found in sagittal sections of the contralateral hippocampal formation impregnated according to the method of NAUTA and GYGAX (1954).

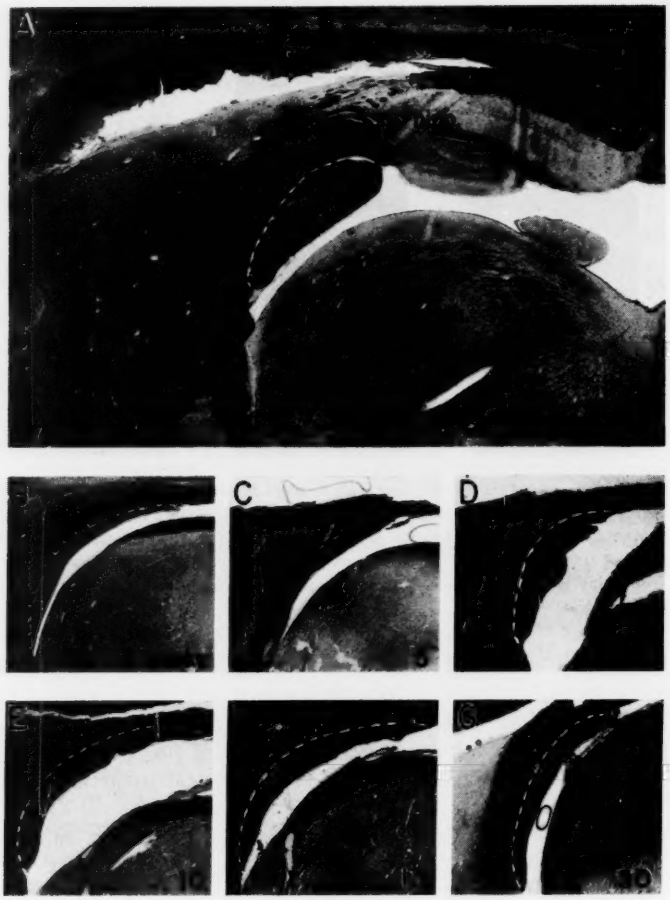

Fig. 8. *A. Topography of the ventral psalterium.* — Photomicrograph of a midline sagittal section through the ventral psalterium and neighbouring structures in the rabbit. Staining of phospholipids according to BAKER (1946).

Abbreviations:

c. a.	anterior commissure	hipp.	hippocampus
corp. call.	corpus callosum	ps. d.	dorsal psalterium
dienceph.	diencephalon	ps. v.	ventral psalterium.
f.	fornix		

B-G. Development of myelin within the ventral psalterium with increasing age in kittens. — Photomicrographs of midline sagittal sections of kittens, aged from 3 to 50 weeks. Sections stained according to the method of BAKER (1946). Dotted lines indicate border of ventral psalterium.

Development of the myelinization of the ventral psalterium fibres. — Of the four methods employed for the determination of myelin (PAL 1886, WOELCKE 1942, HÄGGQVIST 1936, BAKER 1946) the most sensitive for the determination of small amounts of myelin in the ventral psalterium was found to be the histochemical technique for detection of phospholipids — the acid haematein method — as developed by BAKER (1946). Sagittal sections of the ventral psalterium from cats of different ages, ranging from 3 to 50 weeks, were treated according to this method. Preferably, the brains of those kittens in which physiological observations had also been made were employed. At an age of 3 weeks, no trace of phospholipid material could be detected within the ventral psalterium (Fig. 8 B). This was not due to failure of the method, since the stria medullaris and the optic tracts showed great amounts of myelin in the same sections. In 4—5 weeks old kittens phospholipid material appeared, especially in the caudo-dorsal part of the commissure, leaving the ventralmost part free (Fig. 8 C). With increasing age the amount of phospholipids increased and reached the adult level at an age of about 12 weeks (Fig. 8 C—G). The area within the ventral psalterium in which myelinated fibres were observed increased correspondingly. The extension took place from the caudo-dorsal towards the ventral end, but here a little region remained unstained, even in the adult animals.

The degree of myelinization of the ventral psalterium fibres in adult animals. — Sagittal midline sections from the brains of rabbit and cat were treated according to the method of HÄGGQVIST (1936). All clearly visible axons of the ventral psalterium had sheaths which were indistinguishable from the myelin sheaths of the thicker fibres in the optic and pyramidal tracts with regard to stain and density. However, since the psalterium fibres are extremely thin, the determination of a potential myelinization of the finest fibres seems impossible with light microscopy. Sections of the ventral psalterium from all three species, stained according to the method of SCHULTZE (1906), also demonstrated a distinct sheath around fibres with a total diameter as small as 0.5 μ. However, this method cannot be used to determine the degree of myelinization since it stains only the sheaths and not the axons. Therefore, unmyelinated axons will possibly escape recognition with this method. Two modifications of the Weigert method (PAL 1886, WOELCKE 1942) stained fibres with diameters above 2 μ only, and consequently, gave no information regarding the degree of myelinization.

Fibre diameter. — Fig. 9 shows the results of the measurements of the total diameter of the fibres of the ventral psalterium in cat, rabbit and rat. The best results were obtained with the method of SCHULTZE (1906) (Fig. 10) and with phase contrast microscopy. With the latter method, the measurements were made from photomicrographs only (except in the rat) while with the former method the fibres were in addition measured directly from the sections by the use of an eye-piece micrometer.

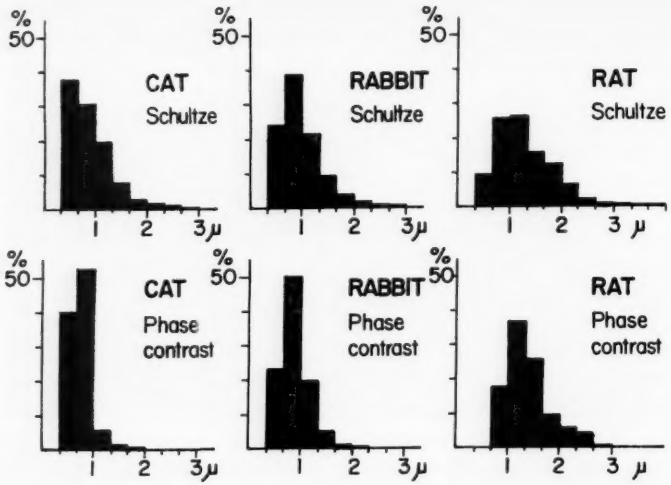

Fig. 9. *Histograms of ventral psalterium fibres in cat, rabbit and rat.* — Ordinate: Number of fibres in per cent of the total number. Abscissa: Fibre thicknesses in thirds of a micron. Upper three diagrams show results obtained from sections stained according to SCHULTZE (1906), lower three from phase contrast microscopy. All values obtained from photomicrographs of the sections.

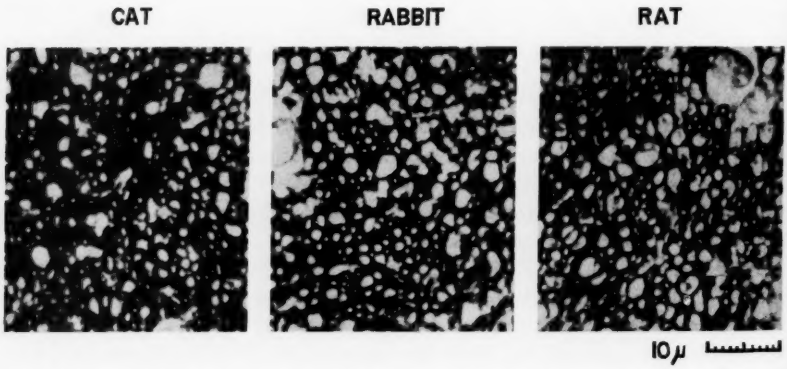

Fig. 10. Representative samples of midline sagittal sections of the ventral psalterium in cat, rabbit and rat stained according to the method of SCHULTZE (1906), an osmium impregnation technique.

Table III. Fibre content of the ventral psalterium in cat, rabbit and rat

Species	Area of the ventral psalterium, mm²			Number of fibres per 100 μ^2		Total number of ventral psalterium fibres
	total	fibre-rich	fibre-poor	fibre-rich	fibre-poor	
Cat	2.02	1.45	0.57	66.5	40.5	1,191,000
Rabbit	0.99	0.67	0.32	72.2	44.7	625,700
Rat	0.49	0.40	0.09	82.1	41.1	365,500

The difference between the photographical and direct microscopical measurements were fairly small. Fig. 9 is based on values obtained from photomicrographs.

In the more homogeneously arranged area, lying caudo-dorsally in the psalterium, the amount of thicker fibres (around 2 μ) was somewhat greater than in the rostral or ventral parts. Silver impregnated sections (BODIAN 1937, NAUTA 1950) were tried, but were found to be unreliable for the measurement of fibre diameters. Within the fibre bundles the impregnated axons appeared as small solid dots of only 0.2—0.3 μ diameter, while the fibres in the more loosely packed areas between the bundles appeared as solid dots or open rings with diameters from 0.2 to 2.0 μ. Such discrepancies could not be detected with the method of SCHULTZE (1906) or by phase contrast microscopy.

The ratio axon diameter/total diameter, as measured in Schultze sections, was about 0.6 for the thinner fibres (around 0.5 μ) and about 0.9 for the thicker ones (1—3 μ).

Total number of fibres in the ventral psalterium. — The total number of fibres in the ventral psalterium in cat, rabbit and rat may be read from Table III. In addition, the table shows the number of fibres per 100 square micron in the major area of the psalterium consisting of densely packed fibres, and in the smaller caudo-dorsal part with a more sparse fibre content. This area, with a lower density of fibres, is identical with the region in which the greatest amount of the thicker fibres (2—3 μ) were found. In the rostrally situated bundles the density of the fibres was somewhat higher than in the rest of the commissure, but the difference was small.

Discussion

Localization of interhippocampal fibres in the fimbria. — The physiological and the anatomical methods employed gave evidence that the interhippocampal fibres are located in the fimbria near CA3. This finding is in conformity with the results obtained by ALLEN (1948) in a MARCHI study in the dog, and with

the observations of CRAGG and HAMLYN (1957) using both electrophysiological and histological methods in rabbits.

Conduction velocity and fibre diameters. — The figure found for the conduction velocity of the interhippocampal fibres in rabbit (5.6 m/sec) is only half the value (12.5 m/sec) presented by CRAGG and HAMLYN (1957). Control experiments have shown that the lower value for the conduction velocity observed by the present author is not due to low temperature, drying or damage to the fibres, or to incorrect time marking. The discrepancy can probably be explained by the different methods employed. CRAGG and HAMLYN (1957) gave only an estimate of the distance between the stimulating end recording electrodes. Further, they did not state to which point of the potential the latency was measured. However, in the present paper even the use of the crest of the initial positive phase of the spike as the reference point did not increase the velocity in rabbit to more than 6.5 m/sec (mean value), still only half the figure given by CRAGG and HAMLYN (1957). Further, measurements of the potentials from their publication give latencies of the same order of magnitude as those of the present study with comparable electrode locations. The discrepancy may perhaps be explained by different methods of estimation of the interelectrode distance. In the opinion of HURSH (1939), the use of several recording points and plotting the latencies against the respective conduction distances is a better method than the use of one recording point only.

The measured conduction velocities of the interhippocampal fibres are low. This must be ascribed to the preponderance of thin fibres and the absence of thick axons in the ventral psalterium.

Fibre diameters. — The fibre spectra of the ventral psalterium in cat, rabbit and rat are dominated by thin fibres (less than 1.5 μ). On account of the limit of resolution of the light microscope, only fibres with diameters greater than one third of a micron could be measured. For this reason, and because of the difficulties involved in exact measurements of the thinnest fibres visible, the data regarding the percentage value of each fibre thickness category are not truly representative. Probably, the number of smallest fibres has been under-estimated. The figures are also not exact with regard to the absolute values of the fibre diameters. This is mostly due to distorsion and shrinkage during dehydration. The use of buffered osmium tetroxide for fixation preserved the tissue well, and according to BAHR, BLOOM and FRIBERG (1957) the final shrinkage in paraffin is 9—14 per cent. GASSER (1950) observed a swelling of peripheral axons of 2—3 per cent following osmium fixation. However, during dehydration, the latter author noted shrinkage of between 20 and 30 per cent. It is most likely that a shrinkage of similar magnitude also occurs in the central nervous system. Some information about the absolute values of the fibre diameters may be derived from sections studied under phase contrast. These were quickly frozen, cut and kept in the animal's

own plasma. This procedure probably did little harm to the tissue. The only serious trouble which might have been anticipated, tearing of the tissue due to the formation of ice crystals, was not observed. However, the data from phase contrast sections do not differ greatly from those obtained in osmium treated sections. The greatest disadvantage of the phase contrast method was the difficulty in obtaining frozen sections thinner than 10 μ.

Going from cat to rabbit and then to rat, the histograms of Fig. 9 show a change towards thicker fibres. The species difference most clearly seen is the higher proportion of fibres in the range from 1.0 to 3.0 μ in the rat compared with those from the rabbit and cat. These differences are in agreement with the different conduction velocities observed in the three species.

The presence of one peak only in each histogram is in accordance with the appearance of only one spike in the fibre potential. Usually, the conduction velocity of a fibre group refers to the thickest fibre category of that group (HURSH 1939). However, such calculation demands monophasic recording of the fibre activity. Therefore, in the absence of distinct peaks in the fibre spectrum, the calculation of a conversion factor between the conduction velocity of the interhippocampal fibres and their total diameter is less exact than the corresponding factor for peripheral nerves. To make an estimate of the order of magnitude of such a conversion factor, the mean value of the fibre diameters may be used. The latency of the diphasic spike, measured to the point of intersection with the baseline, may be accepted as the average latency for the fibres. Calculating on a basis of the mean conduction velocities (3.3, 5.6 and 7.5 m/sec) and the average total fibre diameter (0.6, 0.9 and 1.2 μ), the conversion factors will be 5.5, 6.2 and 6.3 for cat, rabbit and rat, respectively. These figures are in agreement with the factor of 6.0 for A fibres of peripheral mammalian nerves (HURSH 1939) and for B fibres of mammalian preganglionic nerves (GRUNDFEST 1939).

Conduction velocities as low as those determined here for the interhippocampal fibres have been noted in various central fibre tracts. WOOLSEY and CHANG (1947), in a study of antidromical impulses in the pyramidal tract of rabbits, found velocities from 2 to 25 m/sec. The slowest conduction velocity probably represents the propagation along the thinnest fibres, which in the rabbit pyramid are below 1 μ (LASSEK and RASMUSSEN 1940). A conduction velocity of about 7 m/sec for the septo-temporal fibres through the fimbria in cats was reported by STOLL, AJMONE-MARSAN and JASPER (1951).

The comparatively short duration and the great amplitude of the negative phase of the diphasic spike, as well as the marked constancy of this potential, indicate a high degree of synchronization of the interhippocampal volley, a rather astonishing finding in view of the slow conduction velocity.

The observation of an increasing number of the ventral psalterium fibres with an ascent along the phylogenetic scale is paralleled by the calculations of the total number of fornix fibres in macaque (SIMPSON 1952) and man

(DAITZ 1953). By comparison at corresponding levels of the fornix, the fibre content of the human fornix was about double that of the macaque.

An increase in the number of fibres in a tract within the brain may presumably be interpreted as a sign of an increasing functional role of the structure in question (SZENTÁGOTHAI-SCHIMERT 1941, DAITZ 1950). The results of SIMPSON (1952), DAITZ (1953) and those of the present investigation lends, therefore, support to the assumption of an increasing functional role of the hippocampus-fornix system with an increasing phylogenetic level, whatever this function or functions may be.

Conduction velocity and myelinization. — It appears that the physiological observations on the development of the conduction velocity of the interhippocampal fibres in the kitten may be correlated with the myelinization of these axons. The first trace of phospholipid material in the ventral psalterium is found at the age when the first sign of fibre activity can be detected by physiological methods, and the increase in myelinization parallels the increase of the speed of propagation. Both processes reach their final stages when the kittens are about 12 weeks old. However, ULETT, DOW and LARSELL (1944) observed that the transcallosal response in rabbits appeared at an age between the first and fifth day of life. At this stage no trace of myelin could be detected by a modification of the PAL-WEIGERT method. This finding suggests that the relation between the myelinization and the onset of conduction ability may vary in different central tracts. Furthermore, DEL-CASTILLO and VIOZO (1953), in a study of conduction in sciatic nerves in chick embryos, observed propagated action potentials from the 9th day of incubation. At this stage no trace of myelin could be detected by the WEIGERT method or by osmium staining. This observation makes it likely that the peripheral fibres differ from the interhippocampal fibres with regard to the relation between the onset of conduction and myelinization.

Most likely, the increasing conduction velocity is due to the increase of the myelin sheaths around all psalterium fibres. But, the possibility exists that the myelinization of the thicker fibres alone is responsible for the increase in the speed of propagation. The last problem is of essential interest. Do all interhippocampal fibres mature simultaneously, or do different categories become myelinated at different times?

The observation of only thin interhippocampal fibres which mature later than other fibre tracts containing thicker fibres, is in accordance with the view held by SZENTÁGOTHAI-SCHIMERT (1941). From a study of various fibre tracts in the central nervous system, the latter author reached at the conclusion that an early myelinization resulted in the development of thick fibres, while a late myelinization gave thin fibres. The present observations are in agreement whith this view, as the myelinization started in the caudo-dorsal part of the ventral psalterium, the area in which the thickest fibres are most abundant.

Conduction velocity and temperature. — As anticipated, the conduction velocity of the interhippocampal fibres decreased when the environmental temperature of the fibres were lowered. The temperature coefficient for the conduction velocity was lower for the thin interhippocampal fibres than it is for A fibres of peripheral nerves. However, the discrepancy is not great. In frog sciatic nerve the Q_{10} for conduction velocity ranges from 4.1 to 1.4 in the temperature interval 5—30° C (GASSER 1931). Between 20 and 30° C it decreased from 1.7 to 1.4. In mammalian C fibres the Q_{10} for conduction velocity has an average of 1.3 between 20 and 30° C (GASSER and GRUNDFEST 1939). In the present work the Q_{10} for conduction velocity along interhippocampal fibres was about 1.4 in the corresponding temperature range (Table II). Thus, it seems as if the temperature coefficient for the speed of propagation in the nerve fibres mentioned is little influenced by the thickness of the axons or their myelinization. This suggests that the fundamental processes involved in impulse conduction is essentially the same for the three types of fibres.

It is not unlikely that the value given for the temperature coefficient for the interhippocampal conduction velocity needs a small correction. The temperature was measured on the surface of the fimbria and not within this structure. It may be assumed that the interior of the fimbria maintains a somewhat higher temperature than the surface. This may be predicted by the quick rise of the surface temperature following cessation of the cool paraffin irrigation. The presence of a higher temperature within the fimbria compared with the surface would infer that the reported temperature coefficient is too high. However, since the thickness of the fimbria is only about 1 mm, and since a similar temperature coefficient was found in experiments with local cooling as in those with general body cooling, the error is presumably small.

Temperature coefficients do not appear to have been measured for conduction velocity in other fibre tracts within the central nervous system. BISHOP, JEREMY and LANCE (1953 b), in a study of the pyramidal tract in cat, reported that a decrease of the temperature from 37 to 31° C slowed the impulse propagation in thick as well as in thin fibres. Calculating from their figures, the Q_{10} for conduction velocity of the pyramidal fibres is found to be of the same order of magnitude as that reported in the present study.

Impulse conduction and anoxia. — In accordance with the conditions in peripheral nerves of frog (HEINBECKER 1929) and of lobster (WRIGHT 1947), the conduction velocity of the interhippocampal fibres decreased with increasing duration of the anoxia. The conduction velocity of the interhippocampal fibres reached half the normal value in about 30 minutes of anoxia. The corresponding values for the lobster nerves was 72 minutes (WRIGHT 1947), and for the frog nerves 2—4 hours (HEINBECKER 1929).

The time from the onset of the anoxia to the abolition of all fibre activity was shorter for the interhippocampal fibres than for the peripheral nerve fibres. Mammalian A fibres conduct for about 30 minutes during anoxia

(GERARD 1930, LEHMANN 1937, FRANKENHAEUSER 1949), while the corresponding time for frog A fibres is about $1^1/_2$ hours (GERARD 1930). However, the time to anoxic abolition of the interhippocampal fibre activity was closer to that of isolated spinal axons. Fibres, having properties like A fibres, in dissected funiculi from the spinal cord of cats showed unchanged conduction for about 4 minutes of anoxia, then the amplitude of the fibre potentials decreased, and at about 9 minutes the conduction was completely abolished (RUDIN and EISENMAN 1954). These values are close to the corresponding figures for the interhippocampal fibres.

In those experiments where the duration of the anoxia necessary for abolition of the fibre activity exceeded 10 minutes, the persisting conduction was probably partly due to direct oxygen uptake of the fibres from the dissolved air in the paraffin pool. This assumption is proposed on the basis of the similar findings for the saphenous nerve in rabbits (FRANKENHAEUSER 1949).

Comparison of the interhippocampal fibres with the B fibres of the autonomic system. — The B fibres of the preganglionic nerves in the sympathetic system conduct at velocities similar to the interhippocampal fibres, namely from less than 3 to 14 m/sec (GRUNDFEST 1939). The B fibres have an absolute refractory period of 1.1—1.5 msec, and total diameters less than 3 μ, and they are myelinated. The duration of the spike is 1.2 msec, and the period of latent addition is 0.2—0.3 msec. The temperature coefficient for the conduction velocity is 1.3 in the span 20—30° C. All these properties correspond to those of the interhippocampal fibres. These findings suggest that myelinated fibres within the central nervous system, of the same thickness as B fibres, have similar properties as the latter. This supports the assumption that the fundamental processes for impulse conduction are essentially the same in central nerve fibres and in the peripheral nerves, *e. g.* the conduction is presumably saltatory also centrally. The question of the presence of RANVIER's nodes or comparable structures within the fimbria, is, therefore, of particular interest and should be studied in future investigations.

Summary

1. Interhippocampal fibre potentials were evoked by stimulation of a point at the fimbrio-hippocampal border and monopolar recording from points lying along the corresponding contralateral border. The fibre activity was represented by an initial diphasic spike of the response obtained. The spike had a smaller amplitude near the midline compared with points situated more laterally, suggesting that the fibres make a ventral bend in order to cross the midline. This ventral bend was also found by histological methods. The total number of fibres in the ventral psalterium was approximately doubled going from rat to rabbit, and again from rabbit to cat. The number

of the interhippocampal fibres and their diameters show an inverse relationship in the three species studied.

2. The conduction velocity of the interhippocampal impulses was found to be 3.3 m/sec in cat, 5.6 m/sec in rabbit and 7.5 m/sec in rat. Correspondingly, the mean fibre diameter was found to be greater in rat than in rabbit, and smallest in the cat.

3. In kittens, the first signs of interhippocampal fibre activity were observed at a body weight of 0.3—0.4 kg, corresponding to an age of 2—3 weeks. The conduction velocity increased with increasing body weight, reaching its adult value at a body weight of about 1.0 kg, corresponding to an age of about 12 weeks. The onset and increase of conduction velocity were paralleled by the onset and development of myelin in the ventral psalterium, as determined by a sensitive method for detection of phospholipids.

4. The conduction velocity decreased with lowering of the temperature. The temperature coefficient (Q_{10}) was found to be from 1.5—1.2 between 20 and 45° C, being similar to that of conduction velocity in A and B fibres of peripheral nerves.

5. The conduction velocity decreased with increasing duration of the anoxia.

6. The absolute refractory period was 1.2—1.3 msec in all three species, and the relative refractory period 4—5 msec. The period of latent addition was 0.3 msec.

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Effects of RNA on Early Chick Embryos Cultivated in Vitro

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Abstract

EMANUELSSON, H. *Effects of RNA on early chick embryos cultivated in vitro.* Acta physiol. scand. 1960. *48.* 352—363. — The object of the present investigation has been to find out whether pure RNA can exert a stimulating action on the morphogenesis of early chick embryos. The experiments have been performed on 16 to 18-hour-old embryos cultivated in vitro. RNA from 1) area opaca of 2-day-old chick embryos 2) from heads of 6-day-old chick embryos and 3) from yolk-sacs of 6-day-old chick embryos was tested. — After 24 hours' cultivation in vitro a marked growth and differentiation occurred in embryo-regions homologous to those from which the RNA had been isolated. Other regions, however, were rather retarded in their development and displayed disturbed mitoses. Mitotic counts demonstrated an increased mitotic activity in the growth-stimulated embryo-regions. In the latter a slight increase in the percentage of anaphases + telophases also was noted. — Thus it appears that the pure RNA in itself has the capacity of stimulating growth and differentiation in chick embryos, the localization of the effect being dependent, however, on the origin of the RNA used.

As the result of their grafting experiments on early embryos several investigators have arrived at the conclusion that among other effects RNA exerts an inducing activity on embryonic morphogenesis. Most investigators (BRACHET 1957 p. 402 *et seq.*) are of the opinion that it is in combination with protein that RNA can act as an inductor, but there are also indications (NIU 1956) that RNA itself can fulfil this mission.

RNA from chick embryos has been tested as inductor in newt and axolotl embryos (TIEDEMANN and TIEDEMANN 1956), but very weak inductive effect was reported.

When CHÈVREMENT and FIRKET (1952) investigated the effect of pure RNA in low concentrations on tissue cultures, they found a stimulating effect from RNA on cell-multiplication; but this effect was mainly to be found in those cultures where conditions were suboptimal with reference to embryo extract, of which only traces had been added to the medium.

DEOTTO (1956), however, who has studied the effect of pure RNA on various materials (hanging drop cultures of chick fibroblasts, sea urchin embryos, plant roots etc.), states that the general effect of RNA when added in low concentrations is inhibition of cell-multiplication and morphogenesis. The same author has also made experiments in which RNA-solution was injected into the amnion of chick embryos, but unfortunately crucial importance could not be attached to the latter experiments owing to the high mortality among the embryos caused by the technique employed.

In the present investigation the object for the RNA-treatment has been isolated chick embryos at the primitive streak stage. The reason for using embryos of so early age has been that these — still being in the gastrula stage — are especially sensitive to external stimulus. Accordingly it was to be expected that if the various isolated ribonucleic acids really exert any special effects on embryogenesis in the chick, these ought to become particularly manifest at this very stage.

Material and Methods

The experiments were performed on chick embryos (White Leghorn), 16—18 hours old according to the Hamburger system.

The embryos were at this stage transferred into Carrel flasks and cultivated there for exactly 24 hours on a modified Spratt's minimum medium to which RNA was also added.

Control embryos were cultivated on the pure minimum medium alone.

Modified Spratt's minimum medium:
(*cf.* SPRATT 1948, HOWARD 1953)

NaCl	0.650 per cent
KCl	0.033
$CaCl_2 \cdot 6\ H_2O$	0.031
$Na_2HPO_4 \cdot 2\ H_2O$	0.007
KH_2PO_4	0.003
$NaHCO_3$	0.055
Glucose	0.850
Agar	0.420

Buffer components are dissolved separately and the resulting solution is saturated with CO_2 before addition of other components.

Previous investigations (EMANUELSSON 1958) have proved this minimum medium to be suitable for the cultivation of early chick embryos. Thus explanted embryos when cultivated on it for 24 hours undergo a normal differentiation, only slightly slower than in embryos not removed out of the egg.

Addition of RNA was carried out by first dissolving it in a solution made up from

Table I. Base ratios of isolated ribonucleic acids. All ratios are expressed on a molar basis with respect to adenine

RNA	Guanine	Adenine	Cytosine	Uracil
isolated from				
Area opaca (Embryo: 40—60 h)	2.28	1.0	1.63	1.58
Yolk-sacs (Embryo: 6 days)	1.89	1.0	1.65	1.0
Embryo heads (Embryo: 6 days)	1.82	1.0	1.41	0.81

the buffer components of the minimum medium. This solution was then sterilized by filtration through a Seitz filter and finally mixed with the other components, which had been sterilized by boiling just before. The pH of the complete medium was 7.4.

Preliminary experiments had shown that added RNA gave effect in concentrations from 0.06 to 0.27 mg/ml cultivation medium. In the present investigation the concentration was usually held at 0.17 mg/ml. The RNA was always used immediately after it had been isolated.

Three different ribonucleic acids were investigated. They were isolated from respectively 1) area opaca of chick embryos, 40—60 hours old 2) yolk-sacs of 6-day-old chick embryos 3) heads of 6-day-old chick embryos. Isolation of the RNA was made according to the method of KIRBY (1956), but some modifications, as proposed by NIU (private communication) were applied. Thus the selected tissue was rapidly chilled to 2° C (freezing was avoided) and was homogenized at the same temperature in 0.9 per cent NaCl (as opposed to distilled water in the original method) before the addition of phenol.

Furthermore, when homogenizing the tissues it was arranged that the resulting concentration of RNA in the homogenate was maintained at about 40 μg/ml.

The isolated ribonucleic acids were analyzed to ascertain their base composition. Cf. Table I. For that purpose four separate samples from each RNA were hydrolyzed in N-HCl for 1 h at 100° C and then chromatographed on filter paper. Solvent system: isopropanol — conc. HCl — water (98: 25: 28 by vol.) All samples proved to be in good agreement. No traces of proteins or protein derivatives in the ribonucleic acids were observed in these chromatograms.

In all experiments the explanted embryo has been a quadrangular area, dissected out of the blastoderm, and has included the former part of the primitive streak with Hensen's node. In Fig. 1 the extension of an explanted 18-hour-old embryo is outlined.

The cultivation was carried out at 37.0° C $\pm$ 0.5°. Afterwards the explants were fixed in Carnoy's fluid and stained whole in Gomori's hematoxylin according to MELANDER and WINGSTRAND (1953). The preparations were finally mounted in Euparal or DPX.

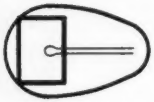

Fig. 1. Schematic drawing of chick embryo of 18 hours of incubation. Heavy lines limit that part of the embryo which is cultivated in vitro.

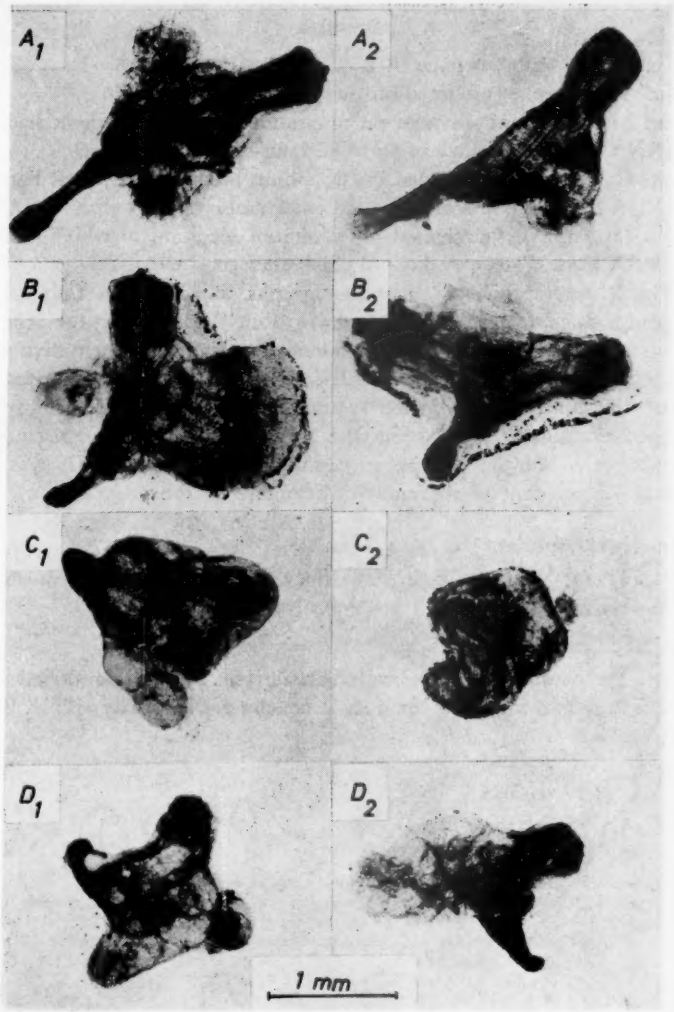

Fig. 2. Chick embryos explanted at the 16—18 hour stage and then cultivated in vitro for 24 hours.
A_1, A_2: Controls, cultivated on the pure minimum medium
B_1, B_2: Embryos, cultivated on minimum medium to which was added RNA from area opaca of 40—60-hour old chick embryos
C_1, C_2: Embryos, cultivated on minimum medium to which was added RNA from yolk-sacs of 6-day-old chick embryos.
D_1, D_2: Embryos, cultivated on minimum medium to which was added RNA from heads of 6-day-old chick embryos.
Gomori hematoxylin. Yellow filter.

Results

The cultivated embryos have been classified in 4 groups, A—D.

Group A: Controls, cultivated on the pure minimum medium.

Group B: Embryos, cultivated on minimum medium, to which had been added RNA from area opaca of 40 to 60 hour-old chick embryos.

Group C: Embryos, cultivated on minimum medium, to which had been added RNA from yolk-sacs of 6-day-old chick embryos.

Group D: Embryos, cultivated on minimum medium, to which had been added RNA from heads of 6-day-old chick embryos.

In Fig. 2 are reproduced microphotographs, showing two embryos from each group. Such microphotographs have been the basis for the schematic drawings in Fig. 3—Fig. 6, which are intended to give a representative picture of the morphology of the explants. All embryos are drawn to the same scale.

About 150 embryos from group A and 70 from each of the groups B—D have been examined. Disregarding the regular occurrence of entirely abnormal embryos in all the groups, successful treatment with RNA was noted for about 75 per cent of the embryos belonging to the groups B-D.

Morphological Observations

After 24 hours' cultivation in vitro the explanted embryos have acquired the following appearances:

Group A:

These, the control embryos, are characterized by well-developed neural tube and brain. In the latter the optical vesicles are distinctly visible. Somites

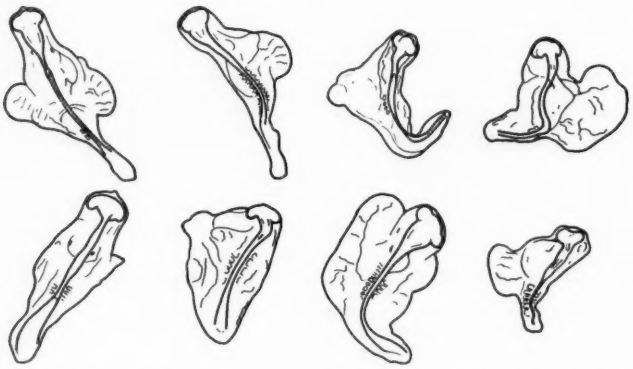

Fig. 3. Schematic drawings of chick embryos, explanted at the 16 to 18-hour stage and then cultivated in vitro for 24 hours on the pure minimum medium. The drawings, which are intended to give a representative picture of the results are based on microphotographs of the same sort as in Fig. 2.

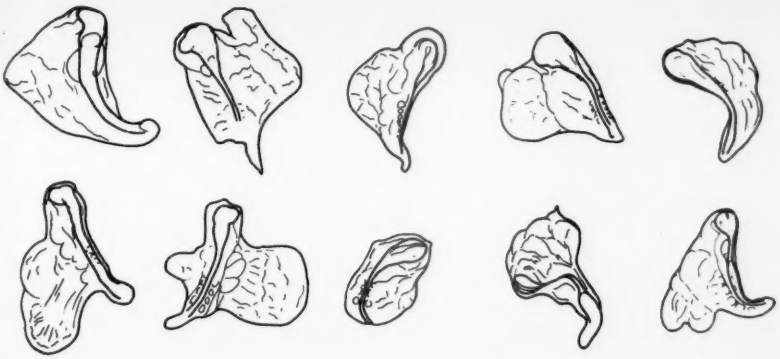

Fig. 4. Schematic drawings of chick embryos, explanted at the 16 to 18-hour stage and then cultivated in vitro for 24 hours on minimum medium to which was added RNA from area opaca of 48—60-hour-old chick embryos. The drawings, which are intended to give a representative picture of the results, are based on microphotographs of the same sort as in Fig. 2.

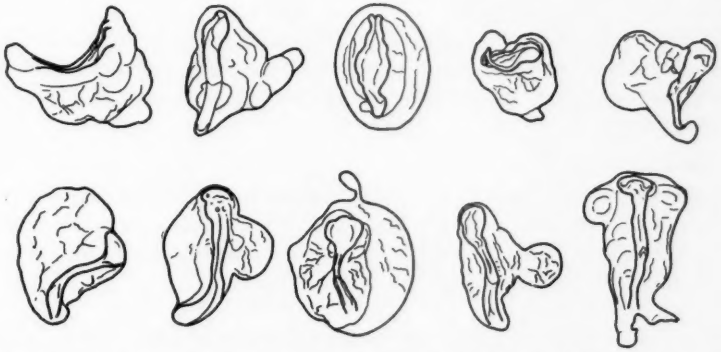

Fig. 5. Schematic drawings of chick embryos, explanted at the 16 to 18-hour stage and then cultivated in vitro for 24 hours on minimum medium to which was added RNA from yolk-sacs of 6-day-old chick embryos. The drawings, which are intended to give a representative picture of the results, are based on microphotographs of the same sort as in Fig. 2.

are always to be found. Outgrowth on either side of the middle part of the embryo — in the future referred to as "marginal outgrowth" — is noted, but it is usually not very pronounced.

Group B:

Compared with the embryos of group A the embryos belonging to the B group are somewhat retarded in their development of the neural tube and the brain. In most cases somites are visible, but sometimes they can be missing.

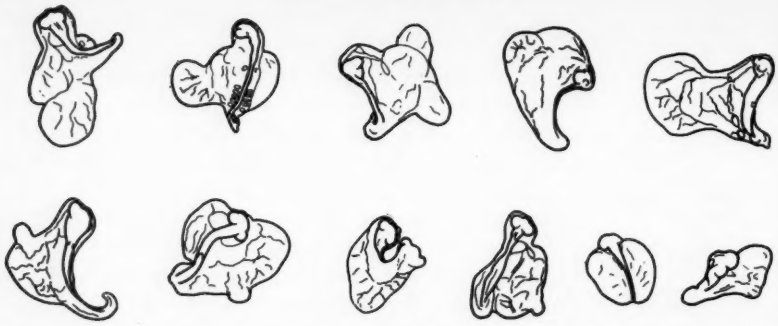

Fig. 6. Schematic drawings of chick embryos, explanted at the 16 to 18-hour stage and then cultivated in vitro for 24 hours on minimum medium to which was added RNA from heads of 6-day-old chick embryos. The drawings, which are intended to give a representative picture of the results, are based on microphotographs of the same sort as in Fig. 2.

The outstanding characteristic of the embryos is the heavy marginal outgrowth.

Group C:

In these embryos the differentiation of the central nervous system is obviously blocked. In most cases visible brain vesicles are accordingly missing. Sometimes somites are developed but they are usually missing. The marginal outgrowth is conspicuous.

Group D:

Generally the embryos of this group are smaller than those from the other groups. A neural tube has developed, but it is rather short. The brain is well differentiated with marked optic vesicles. Somites are mostly missing. A comparatively large marginal outgrowth is noted.

The results suggest that for the explanted embryos added RNA provokes a stimulation of the growth and — at least for group D — also the differentiation of organs in regions, homologous to those from which the RNA was isolated. As for the remaining parts of the embryo-body, however, the visible effect is mostly a suppression of development, *i. e.*, growth and differentiation of these parts are markedly retarded as compared with the controls. The nervous system seems to be particularly sensitive to such suppression.

Mitotic Activity

The morphologic observations above have also been supplemented with estimations of the mitotic activity. In this case it has been of special interest to obtain mitotic indices, *i. e.*, the number of mitoses per one hundred cells, for the head region and for the region of marginal outgrowth respectively. The results are given in Table II.

Table II. Mitotic indices (=number of mitoses per one hundred cells) for RNA-treated embryos and controls. The figures are means of values from corresponding areas of four separate embryos. In each single measurement 1,000 — 2,000 cells have been counted. Investigated areas are marked off in Fig. 7

	Group A	Group B	Group C	Group D
Brain area	4.20 ± 0.33	3.30 ± 0.13	3.73 ± 0.33	6.36 ± 0.89
Area of marginal outgrowth	1.79 ± 0.19	2.44 ± 0.21	1.39 ± 0.04	1.37 ± 0.05

On the whole the values above will confirm the morphologic results. Thus the mitotic indices indicate that for group D there is an increased mitotic activity in the brain area, whereas the reverse condition prevails for the corresponding area of group B and group C.

In the region of marginal outgrowth again there appears for group B a distinct increase of the mitotic activity. Contrary to this corresponding areas for group D present values which suggest a suppression of mitotic activity.

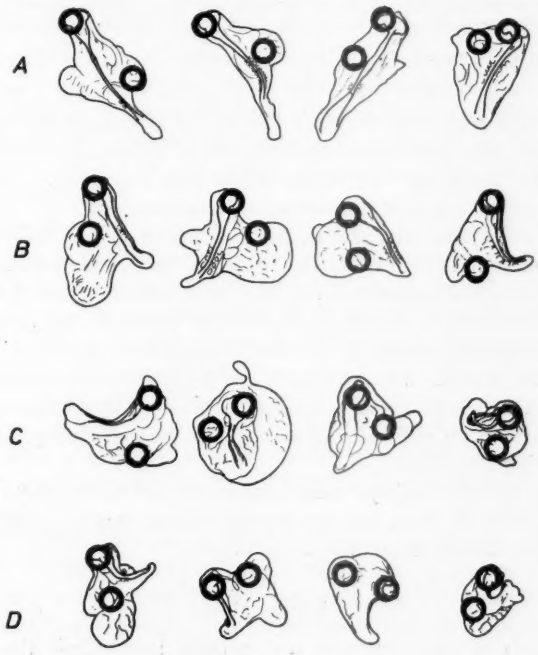

Fig. 7. Schematic drawings showing embryos from the four groups (A—D), which have been selected for estimation of the mitotic activity. Investigated areas are encircled. All embryos are drawn to the same scale.

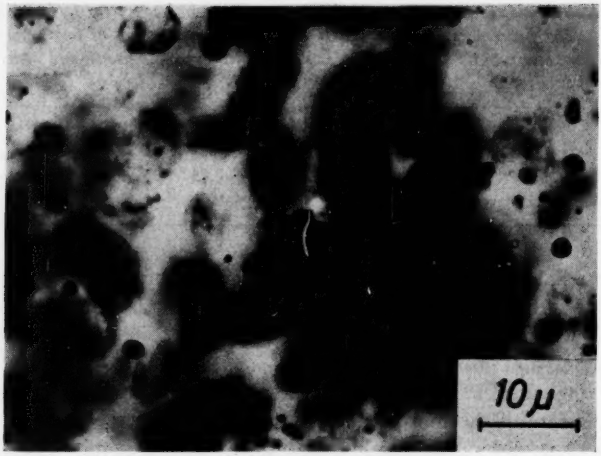

Fig. 8. Microphotograph showing disturbed mitoses from the neural groove of an embryo, cultivated on minimum medium to which was added RNA from yolk-sacs of 6-day-old chick embryos. The large grey oval spots are nuclei. Smaller black globules derive from disintegrated nuclei.
Gomori hematoxylin. Yellow filter.

Finally, it will seem that the values for group C remarkably enough point in favour of a suppression of mitotic activity in the region of marginal outgrowth. In the latter case one would rather have expected an increased cell division, corresponding to the morphologic observations.

As to the mitotic spectrum, *i. e.*, the occurrence of the various phases of mitosis, it is observed that in areas where RNA has stimulated cell-multiplication the percentage of ana- and telophases is somewhat increased, as compared with values from the control embryos. For the latter the percentage of anaphases + telophases is thus 21 ± 5 for the two types of investigated areas, whereas for the brain area of embryos of group D the corresponding percentage is 30 ± 2 and for the marginal outgrowth area of embryos of group B 30 ± 1. Otherwise there is no difference between RNA-treated embryos and controls as regards this percentage.

Inspection of the embryo cells further reveals many disturbed mitoses and karyorrhexis in those embryo regions where morphogenesis had been blocked by the RNA-treatment. *Cf.* Fig. 8.

Discussion

In the study of embryogenesis and protein biosynthesis the problem of control of growth by organ-specific substances holds an outstanding position, and accordingly it has been subjected to extensive experimental investigations.

In 1941 WEISS reported that incorporation of liver and kidney fragments from 6-day-old chick embryos into the area vasculosa of 4-day-old chick embryos provoked a considerable increase in mass of the homologous organ of the latter as compared with controls.

Similar observations have been made by EBERT (1954), who studied the effect of chorio-allantoic grafts of spleen from adult chicks on 9-day-old host chick embryos.

On the other hand, several authors report that addition of small fragments as well as extracts from organs of older chick embryos also can have an inhibiting action on the differentiation of the homologous organs of younger embryos.

Thus WEISS (1952) has shown that extracts of whole chick embryos have an inhibiting action on isolated heart and kidney fragments from embryonal chick cultured in vitro. This inhibition was not found if the extracts were made on embryos from which the homologous organ had first been removed.

DITTMAR, LIPP and AUGSTEIN (1957), who have studied the action of various organ-extracts from chick embryos on fragments of the homologous organs cultured in vitro, report no special effect on the explants from the homologous organs with the exception that extracts from liver had a definitely inhibiting action on the growth of all types of explants.

Apparently it would seem as if the results quoted were conflicting, but as a matter of fact the different effects in the investigations in question are not inconsistent, and they can be satisfactorily explained if one takes into consideration the antagonism prevailing between growth and differentiation during embryogenesis.

As to the biochemical nature of the inducing factors involved in the growth processes just mentioned, there are, however, no definite records.

Now the present investigation actually demonstrates that pure RNA in itself can stimulate morphogenesis in chick embryos and increase cell-multiplication. It will be remembered, however, that those ribonucleic acids which have been made use of here have been isolated according to a method that presumably preserves the properties of the native RNA better than most other isolation methods. Therefore it does not seem improbable that previous failures to prove a stimulating effect on cell-multiplication from pure RNA might have partly been due to the application of unsuitable methods for the isolation of the RNA.

Further, it appears from the present investigation that the stimulating action of RNA affects only the homologous tissues, other tissues rather being suppressed in their development. Considering this, it seems reasonable that the effect of added, non-specific RNA in tissue culture experiments has been inhibition.

When interpreting the results from the chick embryos in the present investigation it will be remembered that the controls with which the RNA-treated

embryos have been compared, have been cultivated on minimum medium exclusively. However — in spite of their complete differentiation — these controls have in fact restricted possibilities to a continued development.

Bearing this in mind, it will perhaps be less surprising that addition of RNA to the minimum medium can give rise to considerable growth changes. Of course, it would have been desirable to carry out a comparison between the RNA-treated embryos here and embryos developing in the intact egg, but that would have been misleading in many respects, as the latter are in possession of an entirely intact area opaca and a complete supply of nutrients. Nevertheless, it is highly interesting that the growth-stimulating effect actually produced by the pure RNA has been specific in such a way that it has been restricted to regions, homologous to those, from which the RNA has been isolated.

As to the mitotic activities, it is a surprise to find a low mitotic activity in the highly developed marginal outgrowth region of embryos of group C, where instead a high activity — as for embryos of group B — was to be expected.

Now when investigating the mitotic activity in a limited area of the region in question all mitoses within this area, irrespective of their distribution at various levels, have been counted, *i. e.*, the area has been limited sideways only. This will imply that the counting might concern different kinds of tissues if they are disposed on each other. One possible explanation to the low activity in question might perhaps be then that the proportion of presumptive yolk-sac tissue in the investigated area — *i. e.*, that very tissue which will be susceptible to stimulation — has not been big enough to produce a general impression of increased mitotic activity.

Finally, the observation that there has been a slight increase in the proportion of ana- and telophases in the RNA-treated embryos is of interest. Thus it indicates the possibility of RNA acting on cell-division by shortening the duration of pro- and metaphases. The other possibility — indication of a prolongation of ana- and telophases — seems less plausible in the present case.

The author wishes to express his sincere thanks to Professor IVAR AGRELL, Head of the Zoophysiological Institute, Lund, for stimulating advice and criticism during the work.

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On the Origin of the Neutral Fecal Sterols and their Relation to Cholesterol Metabolism in the Rat

Bile Acids and Steroids 92

By

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Abstract

DANIELSSON, H. *On the origin of the neutral fecal sterols and their relation to cholesterol metabolism in the rat.* Acta physiol. scand. 1960. *48.* 364—372. — After intraperitoneal administration of a tracer dose of 4-^{14}C-cholesterol to three different groups of rats, intact, bile-duct ligated and bile-fistulated, maintained on a fat-free diet, the excretion of isotope was followed in feces, urine and bile, and the amount of neutral fecal sterols excreted determined. After 12 days subsequent to the injection of 4-^{14}C-cholesterol roughly equal amounts of isotope had been excreted as neutral fecal sterols in all three groups. The average daily excretion of neutral sterols in feces during this period was 3.4 mg in intact, 4.5 mg in bile-duct ligated and 7.3 mg in bile-fistulated rats. In the last-mentioned group 10—15 % of total neutral fecal sterols could not be precipitated with digitonin. During 21 days subsequent to injection of 4-^{14}C-cholesterol into intact rats, the specific activity of the fecal bile acids was calculated to be at least twice that of the neutral fecal sterols.

As part of an investigation in progress on fecal sterols in man and in conventional and germ-free rats, it was considered of interest to study the origin of these sterols and the part played by these in cholesterol metabolism.

These problems have attracted the attention of many investigators over a number of years. BONDZYŃSKI and HUMNICKI (1896) established the presence in feces of coprostanol, normally the major neutral fecal sterol, and suggested that coprostanol might result from bacterial action on cholesterol. This reduction has since that time been assumed to be performed by bacteria, but it was not until 1956, that DAM *et al.* (SNOG-KJAER *et al.* 1956) were able to

achieve the formation of coprostanol from cholesterol *in vitro*. The absence of coprostanol from the feces of germ-free animals (DANIELSSON and GUSTAFSSON 1959) lends additional support to the contention, that coprostanol is a bacterial metabolite of cholesterol.

A minor component of the neutral fecal sterols is cholestanol (COLEMAN *et al.* 1956). This sterol accompanies cholesterol in all tissues in a small (1—5 %) but definite amount (SCHOENHEIMER *et al.* 1930). That cholestanol is found in feces somewhat in excess of this percentage has been explained by the preferential reabsorption of cholesterol from intestinal secretions (SCHOENHEIMER *et al.* 1930). This explanation does not seem to be fully correct as DAM and BRUN (1935) and later others (CURRAN and COSTELLO 1956) have shown that cholestanol indeed is absorbed to a considerable extent, and when given in a tracer dose, to about the same extent as cholesterol (CHAPMAN and CHAIKOFF 1959).

In addition to cholesterol, which constitutes about 10 % of total neutral fecal sterols, rat feces contain other unsaturated sterols, fast-reacting in the Liebermann-Burchard reaction, viz. $\Delta 7$-cholestenol, $\Delta 7$-coprostenol, 7-dehydrocholesterol (WELLS *et al.* 1955; COLEMAN and BAUMANN 1957) and methostenol, 4α-methyl, $\Delta 7$-cholesten-3β-ol (NEIDERHISER and WELLS 1959).

Before the work of SPERRY in 1926—27 on fecal lipids in normal and bile-fistulated dogs, the generally accepted view emphasized the bile as the source of the neutral fecal sterols (SPERRY 1926—27). SPERRY showed that exclusion of bile from the intestine did not result in a decrease in the excretion of non-saponifiable material, results that were corroborated by the work of several other investigators (BEUMER and HEPNER 1929, SCHOENHEIMER and SPERRY 1934).

Subsequent work by SCHOENHEIMER and SPERRY (1934) indicated, that the neutral sterols in feces originated mainly from the cholesterol present in intestinal secretions. BAUMANN and coworkers (WELLS *et al.* 1955, COLEMAN and BAUMANN 1957) demonstrated that the major source of the fast-reacting sterols present in feces was the intestinal mucosa, and suggested, that 7-dehydrocholesterol is hydrogenated to the $\Delta 7$-stenols during its intestinal passage.

Considering the quantitative relationship of the neutral fecal sterols to body cholesterol metabolism, these sterols were long considered to be the major end-products of cholesterol (WEINHOUSE 1943). In 1943 BLOCH *et al.* showed that cholesterol is the precursor of bile acids. The quantitative aspects of this pathway in cholesterol metabolism were investigated in 1952 by several workers (BERGSTRÖM, SIPERSTEIN and CHAIKOFF). In their investigation on the metabolic fate of cholesterol, labeled in C_4 or C_{26} with ^{14}C, SIPERSTEIN and CHAIKOFF (1952) demonstrated, that in intact rats 80—90 % of intravenously administered 4-^{14}C-cholesterol was excreted in feces in 15 days. 90 % of the isotope recovered in feces was present in the acidic fraction. However, when the 4-^{14}C-cholesterol was injected into bile-fistula rats, the major

part of the isotope was excreted in bile as bile acids, and only a small amount in feces, mainly as neutral compounds. On the basis of these results they concluded that endogenous cholesterol is catabolized predominantly to bile acids and only to a minor extent, about 10 %, is excreted as non-saponifiable products.

In the hope of gaining additional information on these problems we have studied the excretion of isotope after administration of 4-^{14}C-cholesterol, and determined the quantity of the neutral fecal sterols in three different groups of rats, intact, bile-duct ligated and bile-fistulated.

Experimental

Throughout these experiments white male rats of the Institute stock, weighing approximately 200 g, were used.

Bile fistulas were prepared in the usual manner. On the animals in the group with ligated bile-duct, both the upper and the lower part of the bile-duct were ligated and the duct between the ligatures was cut.

To these two groups of animals the isotope was injected one day after operation.

4-^{14}C-cholesterol, obtained from Radiochemical Centre, Amersham, England, was prior to use checked for absence of autoxidation products by chromatography. 2 μC4-^{14}C-cholesterol were dissolved in 0.1 ml ethanol and an emulsion prepared by adding 0.9 ml of a 1 % solution of bovine serum albumin in saline. The solution was injected intraperitoneally.

Two days prior to the injection of the cholesterol and during the entire experimental period the rats were fed *ad lib.* a semi-synthetic fat-free diet consisting of 30 % casein, 65 % starch, 5 % salt mixture and vitamins (PIHL, BLOCH and ANKER 1950). The rats were kept in metabolism cages during the experiment.

Analytical procedures

All radioactivity measurements were made in a window-less gasflow counter (Tracerlab. Inc., Waltham, Mass., USA).

Bile. Bile was collected in 24 hour portions in 95 % ethanol. The precipitate was filtered off, and the bile was diluted to a suitable volume for radioactivity determination. Several such 24 hour portions were pooled and the ethanol evaporated. The aqueous residue was acidified and extracted twice with butanol. The combined, neutral-washed butanol-extracts were chromatographed on phase system C (NORMAN 1953) for determination of acidic and neutral sterols in the extract.

Urine. The urine, collected in 24 hour portions, was assayed for radioactivity. In the case of the urine from the animals with ligated bile-duct, it was further worked up as described above for bile for determination of amounts of acidic and neutral products.

Feces. The feces, collected in 24 hour portions, were homogenized in hot water with a pestle and then extracted with boiling 90 % aqueous ethanol for 3 hours twice. The combined ethanol-extracts were evaporated and the residue partitioned between petroleum ether and 70 % aqueous ethanol. Both phases were assayed for radioactivity. The non-saponifiable material from pooled petroleum ether phases was isolated and dissolved in 90 % aqueous ethanol, the sterols precipitated with digitonin and the digitonides filtered off. Only in the case of feces from bile-fistula rats were there significant amounts of isotope in the filtrate. The digitonides were dried to constant weight and the radioactivity of these determined. They were then split with pyridine in the

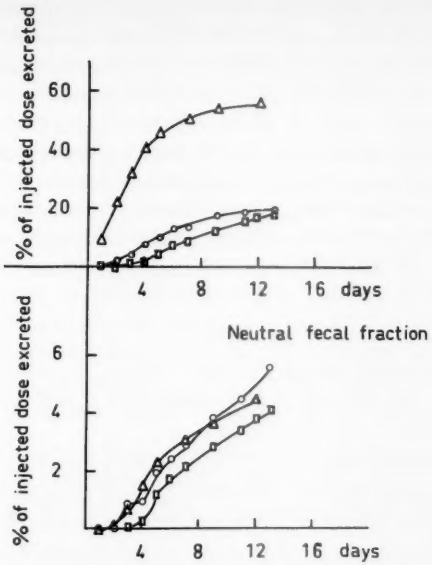

Fig. 1. Cumulative excretion of isotope after administration of 4-^{14}C-cholesterol to intact (□—□—□), bile-duct ligated (○—○—○) and bile-fistulated (△—△—△) rats.
Upper diagram: Cumulative excretion of isotope in acidic fecal fraction of intact rats (□—□—□), in urine of bile-duct ligated rats (○—○—○) and in bile of bile-fistulated rats (△—△—△).
Lower diagram: Cumulative excretion of isotope in the non-saponifiable fraction of feces from intact (□—□—□), bile-duct ligated (○—○—○) and bile-fistulated rats (△—△—△).

usual manner, and after chromatography of the free sterols on aluminum oxide (Merck, Darmstadt, Germany) these were dried to constant weight and assayed for radioactivity.

Results

Fig. 1 shows the cumulative excretion of radioactivity after intraperitoneal injection of 4-^{14}C-cholesterol through the main excretory pathways in the three groups of rats, intact (4 animals), those with ligated bile-duct (2 animals) and those with bile-fistula (4 animals). In the upper diagram is plotted the cumulative appearance of isotope in the 70 % ethanol fraction from feces of intact animals, in the urine from animals with ligated bile-duct and in the bile from bile-fistula animals, and in the lower diagram in the non-saponifiable fraction from feces of all three groups.

After 12 days, as the upper diagram shows, equal amounts of isotope had been excreted in the acidic fraction of feces from intact rats and in the urine of bile-duct ligated ones, while the bile-fistula rats had excreted three times

as much via the bile, a result of increased utilization of cholesterol for synthesis of bile acids, as the formation of bile acids is homeostatically regulated by the amount of bile acids absorbed (BERGSTRÖM and DANIELSSON 1958).

In the lower diagram (Fig. 1) it is seen, that the amount of isotope present in the non-saponifiable fraction of feces is of the same order of magnitude in all three groups of animals, although the actual amount of sterols is somewhat higher in bile-duct ligated and considerably higher in bile-fistula animals, as will be related in more detail below. Thus it is obvious, that also when the flow of bile into the intestine is broken, there is even an increased appearance of neutral sterols in feces.

The labeled excretory products in intact rats.

The amount of neutral sterols excreted in feces by intact rats on a fat-free diet averaged 3.4 mg/day the first 11 days and 3 mg/day the following 10 days.

Of the total radioactivity excreted in 12 days this fraction accounted for 17 %, while the rest, the urine being practically devoid of isotope, was present in the 70 % ethanol fraction of feces, containing the bile acids.

The labeled excretory products in rats with ligated bile-duct

The average daily amount of neutral sterols in the feces from these animals was the first 11 days 4.6 mg and during the following 10 days 5.2 mg. This fraction contained 21 % of the total amount of isotope excreted in 12 days. The 70 % ethanol fraction of feces accounted for 4 % and the remaining 75 % of excreted isotope was present in the urine. An analysis of pooled urines showed, that 95 % of the isotope was in the form of bile acids, and the rest as neutral sterols.

The labeled excretory products in rats with bile-fistula

These animals had an increased daily excretion of neutral sterols as compared to intact rats, 7.3 mg/day during the period of time (varying from 10 to 13 days) it was followed. A notable finding concerning the nature of the neutral sterols in the feces of bile-fistula rats was that 10—15 % of these could not be precipitated with digitonin. Chromatographically this non-digitonin-precipitable fraction behaved as mono-hydroxy neutral C_{27}-sterols, but it appears to be a mixture of as yet unidentified compounds, which do not give a positive Liebermann-Burchard color test.

In the bile, which contained 91 % of total amount of isotope excreted in 12 days, 92 % of the isotope was present in the bile acid fraction and the rest as neutral sterols. The urine and the 70 % ethanol fraction of feces together accounted for 2 % of the amount of radioactivity excreted.

Discussion

The results obtained in the present investigation indicate in agreement with the conclusions of SPERRY (1926—27) and SCHOENHEIMER and SPERRY (1934), that the neutral fecal sterols to a considerable extent are derived from non-biliary sources. Thus rats with ligated bile-ducts excrete approximately the same amount of such sterols as intact rats. Also in bile-fistula rats, as has been demonstrated earlier by several investigators analyzing the amount of non-saponifiable material in feces (SPERRY 1926—27, BEUMER and HEPNER 1929) considerable amounts of neutral sterols are excreted in feces. These facts should not directly be interpreted as to indicate, that the non-biliary excretion is the only source of these sterols, as the experimental approach used, to prevent the bile from entering the intestine, necessarily imposes fundamental changes on the metabolism of cholesterol in the intestinal tract. The obligatory rôle of bile in cholesterol absorption has been demonstrated conclusively (SIPERSTEIN *et al.* 1952). Thus, in absence of bile, there can be no reabsorption of cholesterol excreted by the intestinal wall. The amount of neutral sterols in feces from bile-duct ligated animals, 4.6 mg/day, could then be considered to represent the total daily excretion of cholesterol into the intestinal tract from non-biliary sources. It must be pointed out, though, that a homeostatic mechanism similar to that found in the synthesis of cholesterol (GOULD and COOK 1958) and bile acids (BERGSTRÖM and DANIELSSON 1958) in the liver might operate also in this case. Normally in the process of absorption of cholesterol, it is held up in the intestinal mucosa and mixed with endogenous cholesterol before being delivered into the lymph (BORGSTRÖM *et al.* 1958). Then, when no cholesterol is absorbed, the synthesis of cholesterol in the intestine might be stimulated to operate on a level above normal. In this connection it should also be pointed out, that no attempt has been made to differentiate between the different non-biliary sources, *i. e.* an excretion by the intestinal wall and sloughed mucosal cells. Considering that the whole of the intestine in a 200 g rat contains about 10 mg of cholesterol, it seems likely, though, that the contribution from shed cells to a daily appearance in feces of 3.5—7 mg of neutral sterols is quantitatively less important.

The size of the biliary excretion of cholesterol in an intact rat is not known, but could possibly be estimated to about 3 mg daily. This figure has been calculated from the amount of cholesterol excreted in the bile of a bile-fistula rat the first 6 hours after fistulation, when the bile acid pool is excreted (ERIKSSON 1957). This amount, 0.25—0.3 mg, has been multiplied by 10, *i. e.* the approximate number of times the bile acid pool circulates daily in an intact rat (BERGSTRÖM and DANIELSSON 1958, OLIVECRONA and SJÖVALL 1959).

The non-biliary and the biliary excretion of cholesterol into the intestinal tract would thus amount to 7—8 mg daily, about 50 % of which is reabsorbed in the intact rat, which excretes 3.4 mg/day of neutral sterols in feces. This

reabsorption might primarily affect the biliary cholesterol, as the cholesterol absorption takes place mainly in the upper part of the small intestine (BORGSTRÖM *et al.* 1958, BORGSTRÖM 1960).

The high level of neutral sterols in feces from bile-fistula rats, 7.3 mg daily, might be explained by an effect also on the intestinal synthesis of cholesterol by the increased turn-over rate of cholesterol in such animals, caused by the absence of bile acid and cholesterol absorption (BERGSTRÖM 1959).

The appearance of nondigitonin-precipitable neutral sterols in feces from bile-fistula rats is noteworthy, and preliminary work has indicated, that the presence of these sterols is not due to alterations in the intestinal flora of these animals, as they are present also in animals treated with "intestinal" antibiotics. In this connection it is interesting, that WILSON and SIPERSTEIN (1959) recently have shown that rats fed a diet with 20—30 % corn oil excrete considerable amounts of nondigitonin-precipitable sterols in feces.

Considering the results of the experiments on intact rats in relation to quantitative cholesterol metabolism, we interpret these in a way, which is not fully in accord with the conclusions arrived at by SIPERSTEIN and CHAIKOFF (1952), when these authors state, that only about 10 % of endogenous cholesterol is eliminated as neutral sterols.

Although it is not possible at this moment to accurately determine the quantity of bile acids in feces due to the manifold and often profound changes these undergo by the action of intestinal microorganisms during their intestinal passage (NORMAN and SJÖVALL 1958), a daily excretion of bile acids in rat feces of 4—5 mg can be assumed on the basis of the turn-over rate of bile acids and the size of the circulating pool of these (LINDSTEDT and NORMAN 1956, ERIKSSON 1960). Then, if one from the present experiments on intact rats calculates the specific activity of the bile acids in feces, assuming these to amount to 5 mg/day, and compares that to the specific activity of the neutral fecal sterols, determined to 3—3.4 mg/day, it is found, that during 21 days after intraperitoneal administration of 4-^{14}C-cholesterol, the specific activity of the bile acids in feces is at least twice that of the neutral fecal sterols. This suggests, that the cholesterol synthesized in the intestine, which is one of the immediate precursors of neutral fecal sterols, is not wholly equilibrated with the liver-cholesterol, the precursor of bile acids.

The skillful technical assistance of Mrs. BIRGITTA BENGTSSON is gratefully acknowledged.

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Measurement of Oxygen Tension on the Surface of the Cerebral Cortex of the Cat during Hyperoxia and Hypoxia

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Abstract

INGVAR, D. H., D. W. LÜBBERS and B. SIESJÖ. *Measurement of oxygen tension on the surface of the cerebral cortex of the cat during hyperoxia and hypoxia.* Acta physiol. scand. 1960. *48.* 373—381. — The oxygen tension on the surface of the intact cerebral cortex of the cat was recorded continuously by means of a membrane covered platinum cathode (LÜBBERS and OCKENGA 1958). The stability of the electrode permitted an interpretation of results from different experiments in relative terms. The influence upon the cortical pO_2 of high and low oxygen concentrations in the inspired air was studied. In ordinary hyperoxia only a moderate increase of cortical pO_2 was found. In post-hypoxic hyperoxia the cortical values reached values about 3 times as high. The difference found is interpreted as due to so called reactive hyperemia in the post-hypoxic state.

Polarographic measurement of oxygen tension, or oxygen "availability", with platinum cathodes has been much used in brain physiology since the technique was first introduced by DAVIES and BRINK (1942). Two main types of cathodes have been used, the "naked" type with the platinum exposed to the tissue (DAVIS, MCCULLOCH and ROSEMAN 1944, DAVIES and RÉMOND 1947, MEYER and DENNY-BROWN 1955 *et seq.*), and the "recessed" type with, *e. g.* an agar bridge between the platinum and the tissue (DAVIES and BRINK 1942, BRONK *et al.* 1944).

The recessed type of platinum cathode can be used for absolute but discontinuous measurements of tissue tensions (DAVIES and BRINK 1942). Continuous measurements can be made with the naked cathode, but it will only give relative values. Due to changing catalytic activity of the platinum surface, naked electrodes also tend to be unstable (*cf.* CONELLY 1957). Further-

more, all electrodes, although small, when introduced into the tissue, give rise to injury which influences the measuring conditions.

In the present investigation we have used a membrane covered multiple platinum wire oxygen electrode (LÜBBERS and OCKENGA 1958) of the CLARK-type (CLARK 1956) for continuous measurements in the cat of the local oxygen tension on the intact pial surface of the cortex (here called "cortical surface pO_2"). It will be shown that this type of electrode yielded stable and reproducible measurements over several hours. It was furthermore characterized by a sensitivity and rapidity suited for the problems under investigation. These characteristics of the electrode made it possible to study the influence of hyperoxia and hypoxia upon the cortical surface pO_2 and to interpret the results in relative terms. It was found that, depending upon the preceding state of oxygen supply, oxygen breathing leads to different effects upon the cortical surface pO_2. Thus, in post-hypoxic hyperoxia it reached much higher levels than in ordinary hyperoxia.

Methods

The present series consists of 14 experiments in cats. Four of these were anaesthetized with Nembutal (40 mg/kg i. p.) and ten with ether and Pentothal. In the latter group a brain stem section was made at the collicular level in two cats (*cerveau isolé*), and in four a transection of the spinal cord was made at C I (*encéphale isolé*).

All cats were tracheotomized and, in some cases, a respiratory valve was mounted on the tracheal tube. Gas mixtures could be administered from rubber bags attached to the inlet of the valve. In most experiments a curarizing agent (Flaxedil) was given intravenously and respiration was maintained artificially. The cat's temperature was kept at 38° C (± 1° C) with a heating device.

Polyethylene cannulae were inserted into the femoral artery (for blood pressure recording with an electromanometer) and the femoral vein.

The scull was laid bare over the dorsal surface by means of extirpation of the temporal muscles and a drill hole was placed on one side, usually over the middle suprasylvian gyrus. Pairs of EEG-electrodes for "bipolar" recording were also inserted into the bone. One pair was always placed close to the oxygen electrode.

The oxygen electrode used in the present experiments were modified CLARK electrodes (CLARK 1956, LÜBBERS and OCKENGA 1958). They contained 20—40 platinum wires (25—100 μ each in diameter) melted into a small glass cylinder (outer diameter 1—3 mm) and polished to brightness at the end (MOCHIZUKI and BARTELS 1955, BÜRGER, LÜBBERS and OCKENGA 1957). The polished surface was covered by a 30 μ polyethylene membrane suspended on the electrode housing. The housing contained a saturated potassium chloride solution which was in contact with a silver-silverchloride reference electrode. The polarizing voltage was maintained at — 0.6 V.

Calibration of the oxygen electrodes in Ringer's solution, in blood, or in gas mixtures (at 37°C), all of known oxygen content showed linearity and minimal variability (deviation in 24 hours not more than 2 per cent). Sensitivity in gas was found to be about 5 nA per mm Hg oxygen tension. When the electrode was brought from pure nitrogen into air in one second, or vice versa, the response reached 50 per cent amplitude in 3 sec, 80 per cent in 6 sec, and 100 per cent amplitude in 19 sec. These values refer to the electrodes used in the present investigation. With membranes of different thickness, or other distances between membrane and platinum, shorter or longer response times are obtained.

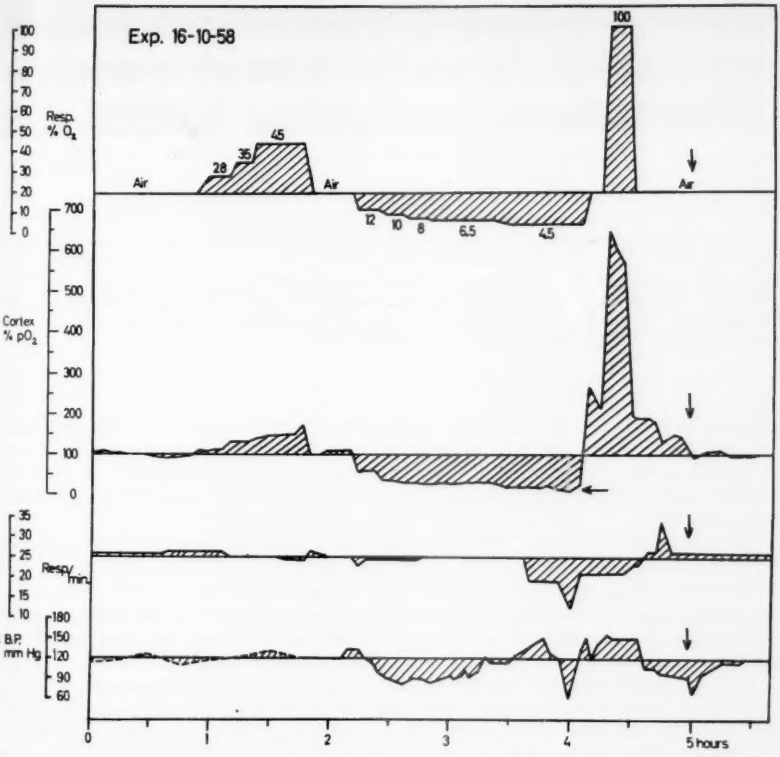

Fig. 1. Cat, Pentothal anaesthesia, Flaxedil and artificial respiration. Diagram illustrating stability and reproducibility of cortical surface pO_2 measurements. From above are shown *per cent oxygen in inspired air, cortical surface* pO_2 in relative percentual terms (see Methods), *respiratory rate, blood pressure* and *time*. Horizontal arrow indicates point where the EEG disappeared for 15 sec. Vertical arrows indicate transection of the spinal cord at C I. Note small variation (± 5 per cent) in cortical surface pO_2 during periods of air administration. Note further the parallelism between cortical surface pO_2 and oxygen content in inspired air. After the periods of hypoxia, however, there followed a period of very high cortical surface pO_2 when air and later 100 per cent oxygen was administered.

Like all membrane covered platinum cathodes without constant distance between platinum and membrane, the electrodes used in the present investigation were sensitive to mechanical pressure. This sensitivity could, however, be diminished considerably by a very tight mounting of the membrane on the electrode housing, and by fixing the glass cylinder containing the platinum wires very close to the membrane. When the membrane was not tightly mounted, artefacts of respiration or brain pulsations were recorded (amplitude less than 3 per cent of resting value).

The current through the electrode circuit was measured by means of a nanoamperemeter (Siemens; input impedance used 400 or 1 330 ohms) from which signals were fed to one of the direct coupled amplifiers (Type 138 A) of an 8-channel Offner EEG

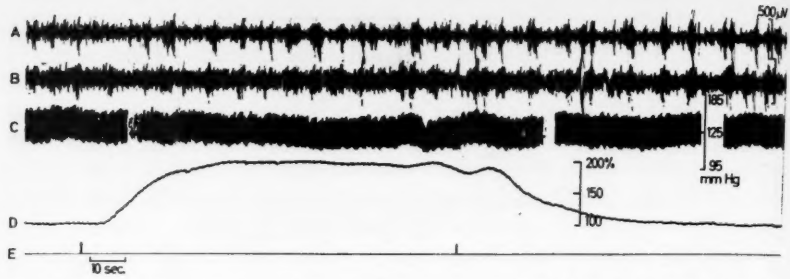

Fig. 2. Cat, spontaneously breathing in Nembutal anaesthesia. Change in cortical surface pO_2 following administration of high oxygen. Records of *EEG* from right sensorimotor and suprasylvian area (*A* and *B*), *blood pressure* (*C*), *cortical surface* pO_2 (*D*) and *signal* (*E*). Between the two signals 100 per cent oxygen was administered.

machine (Type D 3), with circularly writing pens. A pen deviation of twenty mm from the baseline thus gave an ordinate error of 1 per cent, and a time error of 0.8 sec at the paper speed most often used (2.5 mm per sec). Usually 4 channels of the EEG machine were used for EEG recording, one for pO_2 measurements and one for blood pressure.

Measurements of cortical surface pO_2 were made after application of the electrode with its polyethylene membrane through the snuggly fitting drill hole onto the exposed intact pial surface of the cortex. As a rule, areas without larger vessels were selected. Pressure against the cortex was avoided by a holder for the electrode. Vaseline around the electrode when placed in the drill hole minimized gas exchange with room air. The temperature of the cortex was usually 36.0°—37.0° C.

It is not possible at present to calibrate a platinum electrode for *tissue* measurements in absolute terms, since the calibration curve is dependent upon the diffusion conditions prevailing in the tissue. We have therefore chosen to present our data in terms of percentage. The 100 per cent value of cortical surface pO_2 was defined by the mean electrode current in a given experiment at rest under respiration of room air. The reading shortly after the death of the animal in anoxia was taken as zero per cent.

Under controlled respiratory and circulatory conditions and with reasonably constant functional activity (as measured by the EEG), the oxygen electrode recorded stable and reproducible values. This fact is illustrated in Fig. 1 which shows, in an anaesthetized preparation under artificial respiration, almost identical values of pO_2 every time identical respiratory (and circulatory) states were reproduced. During this longlasting experiment the oxygen content of the inspired air was varied within wide limits.

In order to determine the response time of the electrode when measuring *in tissue* it would be necessary to produce a change of oxygen tension of a known form and size in the tissue. With constant tissue respiration such a change would be brought about by changing suddenly the oxygen tension of the blood. There is, however, at present no method available to measure such a change continuously and accurately. It is possible, however, to change the oxygen content of the inspired air and to follow the subsequent change in tissue oxygen tension. This change will, however, be delayed by lung diffusion, circulation time, in addition to the diffusion in the tissue itself. Fig. 2 shows the results of a change from air breathing to inhalation of pure oxygen in a spontaneously breathing preparation under Nembutal anaesthesia.

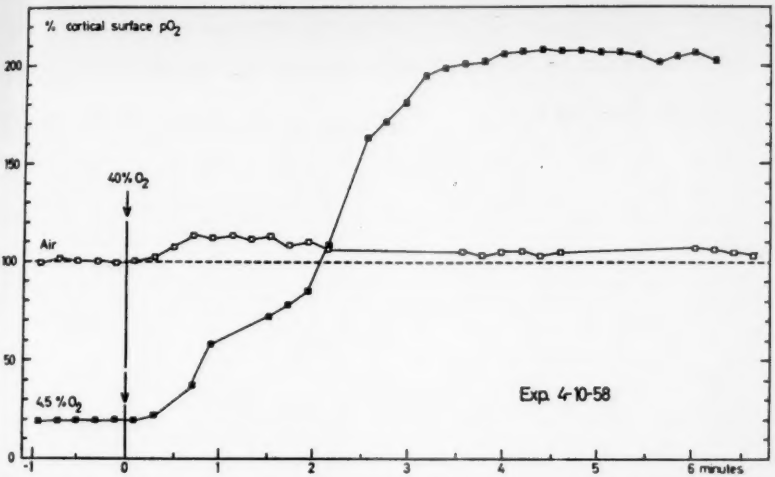

Fig. 3. Cat, *encéphale isolé* prepared under ether and Pentothal, Flaxedil and artificial ventilation. Diagram of changes in cortical surface pO_2 during hyperoxia (40 per cent oxygen in inspired air) preceded by air breathing (unfilled symbols) and hypoxia (filled symbols). Note large increase in cortical surface pO_2 after hypoxia.

The increase of tissue oxygen tension in the cerebral cortex in this and similar experiments started about 5 sec after the onset of the oxygen breathing and reached its full amplitude in about 45 sec. Measuring from the beginning of the rise of cortical surface pO_2, 50 per cent amplitude was reached in 9 sec, 75 per cent in 17 sec, and 100 per cent in 40 sec.

Results

The main results of the present investigation concern changes in cortical surface pO_2 following changes in the oxygen content of the inspired air of the preparation.

When the oxygen concentration was increased from that of room air to, *e. g.* 40 per cent in nitrogen, a higher value was recorded which stabilized in about 1 to 2 min (Fig. 3, unfilled symbols). Similarly, a slow stepwise increase of the oxygen concentration from that of room air up to 100 per cent gave increasing cortical surface pO_2 values, which in a plot showed a curve of moderate slope (Fig. 4, unfilled symbols). Concentrations of oxygen below that of room air gave a somewhat steeper curve.

It was found, however, that hypoxia, or a short period of anoxia, regularly changed the cortical surface pO_2 values when the preparation immediately afterwards was allowed to breathe higher oxygen concentrations. In such experiments hypoxia was induced by having the preparation breath a low oxygen concentration (5—10 per cent) for several minutes (*cf.* Fig. 1) during which

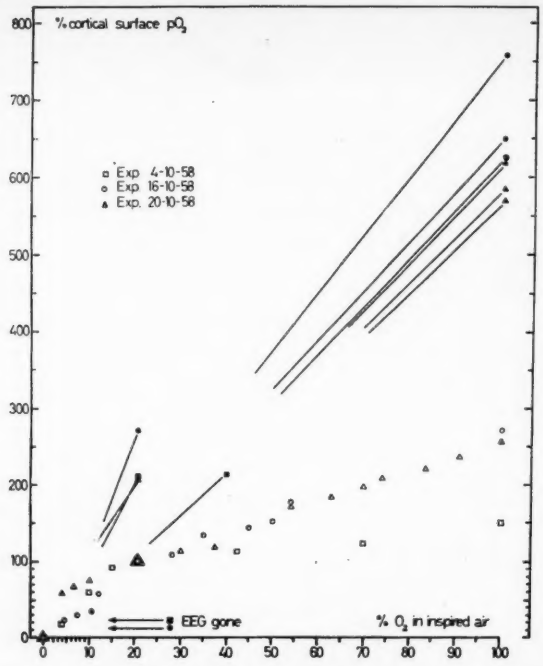

Fig. 4. Cats, *Encéphale isolé* (4/10), Pentothal (16/10) and Nembutal anaesthesia (20/10). In all three experiments Flaxedil was given and artificial respiration used. Diagram of cortical surface pO_2 at different concentrations of oxygen in the inspired air without (unfilled symbols), and with (filled symbols) preceding hypoxia. Note fair reproducibility of cortical surface pO_2 values between the three experiments. Very high values of cortical surface pO_2 were reached in all experiments immediately following a short period of hypoxia (filled symbols). In two of the experiments the hypoxia was carried to a point where the EEG disappeared for 15—20 seconds to be fully restored during the subsequent period of hyperoxia. The level of cortical surface pO_2 at which the EEG disappeared was similar in both experiments (horizontal arrows).

the EEG was observed to remain uninfluenced. Anoxia was produced for very short periods (never exceeding 15 sec) by nitrogen breathing or by hypoventilation. During these short periods there was no electrical activity recorded from the cortex.

When air or higher oxygen concentrations were administered after hypoxia and anoxia, induced as described, the cortical surface pO_2 in 1 to 2 minutes rose to peak values about three times as high as without preceding hypoxia. Such high cortical surface pO_2 values recorded in post-hypoxic hyperoxia in three experiments are shown in Fig. 4 as filled symbols (*cf.* also Fig. 1). In Fig. 3 a comparison is made between a change from breathing air to 40 per cent oxygen on the one hand, and from 4.5 per cent to 40 per cent on the other hand. Continued breathing of high oxygen after hypoxia, however, led

to a slow decrease of the initial high values which ultimately changed into those corresponding to the unfilled symbols of Fig. 4 at the given oxygen concentration. The time taken for this change varied considerably, lasting up to about 30 minutes.

EEG controls in the experiments cited showed that the electrical activity disappeared at a defined cortical surface pO_2 value when the oxygen concentration was diminished. With the short periods of anoxia used, the EEG reappeared at about the same value when air or high oxygen was again administered. The point where EEG disappeared (horizontal arrows on Fig. 1 and 4) represented about 15—20 per cent of the resting value of cortical surface pO_2 with room air respiration. Frequent controls with brain stem stimulations showed that the EEG after the hypoxic periods was still normally reactive, displaying classical arousal patterns. There were no specific differences between the EEG at room air concentration of oxygen and at higher concentrations. A slight increase of amplitude of the cortical potentials was, however, often recorded in post-hypoxic hyperoxia.

The blood pressure only changed moderately during the periods of hypoxia and hyperoxia. As a rule, the variations did not exceed 20 to 30 mm Hg (*cf.* Fig. 1).

If the cerebral vascular resistance was suddenly lowered during hyperoxia, this regularly led to a rapid, though transient increase of the cortical surface pO_2. This increase often reached values close to the high ones in post-hypoxic hyperoxia. Changes of this type were brought about by injection of small doses of Papaverine (1—2 mg in 0.1—0.3 ml) into the central end of the cut lingual artery, or by intravenous injection of Diamox 5—25 mg/kg, or Perfantin (1 mg/kg pyrimido (5,4-d) pyrimidine) (*cf.* SOKOLOFF 1959).

Discussion

The oxygen electrodes used in the present experiments, in contrast to most other types (especially those of the "naked" type), proved to be stable and to yield reproducible values over several hours under controlled circulatory, respiratory and functional conditions. These characteristics of the electrodes have therefore made it possible to relate results from different experiments in relative terms.

Concerning the relationship between the oxygen concentration of the inspired air and the oxygen tension measured on the cortical surface, the main interest should be attached to the fact that different results were obtained in hyperoxia after air breathing, on the one hand, and after a period of hypoxia (or short lasting anoxia), on the other. While, in the first case, the cortical oxygen tension did not rise very much with hyperoxia there was in the latter case, in post-hypoxic hyperoxia, a remarkable increase in cortical surface pO_2.

The difference found between the two groups described might be explained mainly as due to "reactive hyperemia", *i. e.* the increase of cerebral circula-

tion which follows hypoxia or a short period of anoxia. This view is highly supported by the findings with vasodilating agents in hyperoxia described above. "Reactive hyperemia" may reach high levels and outlast the hypoxic period by minutes, as has been shown by NOELL and SCHNEIDER (1942) and KETY and SCHMIDT (1948; see also SCHNEIDER 1953).

As mentioned, the high values of cortical surface pO_2 in post-hypoxic hyperoxia slowly decreased and finally attained low levels similar to those in ordinary hyperoxia (unfilled symbols, Fig. 4). This finding is most likely explained by reduction of the cortical blood flow with the disappearance of the "reactive hyperemia". It is also possible that the well-known cerebral vasoconstrictive effect of high oxygen concentrations (KETY and SCHMIDT 1948) may have enhanced the decrease.

The cerebral oxygen consumption may be lowered substantially, if the oxygen content of the inspired air is lowered for a longer period to a point where the EEG is permanently abolished (NOELL and SCHNEIDER 1942). In the experiments related above, however, the hypoxic periods (and even more so the anoxic ones) were usually short, and the EEG was always fully restored afterwards and responded to brain stem stimulation. We therefore feel justified to conclude that any change in oxygen consumption must have been small in the present experiments and that, therefore, this factor has been of minor importance for the high post-hypoxic cortical surface pO_2 values in hyperoxia. A further evidence for this view is provided by the fact that the cortical functional activity, as judged by EEG, in ordinary, and in post-hypoxic hyperoxia did not show any appreciable differences. It may finally be added that there is no reason to assume that hyperoxia in itself, as used in the present experiments, might have changed cerebral oxygen consumption (KETY and SCHMIDT 1948, LAMBERTSEN *et al.* 1953).

It should be emphasized that the majority of the experiments discussed above were carried out in curarized (Flaxedil), artificially ventilated preparations. This fact excludes that respiratory changes, *e. g.* due to hyperventilation, with ensuing changes in arterial carbon dioxide tension (and hence also in cerebral blood flow) could have been of importance for the main results. Some systemic circulatory reactions such as pressor responses were, however, recorded in the experiments with hypoxia. As seen in the example given in Fig. 1, these were comparatively small and, consequently, their direct influence upon the cerebral blood flow could not have been of greater importance.

Respiration of different oxygen concentrations has been used as a means of calibrating oxygen electrodes for tissue measurements (MEYER and DENNY-BROWN 1955, DAVIES, MCCULLOCH and ROSEMAN 1944). Conflicting results have, however, been reported as to the effect of different amounts of oxygen in the inspired air upon the oxygen tension in the cerebral cortex. For example, oxygen concentrations of 70 or 100 per cent have been found to change the measured cortical value by an amount varying from 10 to 300 per cent (FUR-

LONG and SCHWARZ 1956, CLARK and MISRAHY 1957). In view of the findings reported above it would seem highly unsatisfactory to use hyperoxia of different degrees as a calibration method for platinum cathodes measuring in tissue.

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Autoradiographic Observations on Injected S^{35}-Thiocyanate and C^{14}-Cyanide in Mice

By

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Abstract

CLEMEDSON, C. J., S. SÖRBO and S. ULLBERG. *Autoradiographic observations on injected S^{35}-thiocyanate and C^{14}-cyanide in mice.* Acta physiol. scand. 1960. *48*. 382—389. — The labelled compounds were injected into mice and sections of the whole animals were studied by an autoradiographic technique. The two compounds studied gave an almost identical autoradiographic distribution pattern. This was attributed to the fact that the activity after injection of $KC^{14}N$ was present mainly as thiocyanate. A very high activity was present in the stomach as early as 5 min after injection and it remained higher there than in any other organ during the 24-hours observation period. High activities were also found in the submaxillary salivary gland, the thyroid gland, the walls of the large vessels and the blood.

In connection with investigations on the toxicology of cyanide (CLEMEDSON, HULTMAN and SÖRBO 1954, 1955) it was considered to be of interest to study the distribution of cyanide and its main detoxification product, thiocyanate, in the body. An attempt was made to study the distribution of $KC^{14}N$ by autoradiography but it was soon realized that because of the volatility of hydrocyanic acid (cyanide is mainly present as the free acid at the pH of body fluids), cyanide *per se* could not be detected in the sections. Some radioactivity, however, was actually demonstrated but this must have been due to a nonvolatile conversion product. The latter was tentatively identified as thiocyanate by a direct chemical analysis of the gastric contents. The similarity of the autoradiographic patterns obtained with $KC^{14}N$ and $KS^{35}CN$ suggested that thiocyanate was this nonvolatile product in other parts of the body as well.

Methods

The labelled compounds, $KC^{14}N$ with a specific activity of 2 mC/mM and $KS^{35}CN$ with a specific activity of 13 mC/mM, were obtained from the Radiochemical Centre, Amersham, England.

Each compound was given to five adult male mice (weight about 25 g) and to five pregnant females (weight about 45 g). The latter were in advanced pregnancy and received the compound two days before expected delivery.

The compounds were administered intravenously in a tail vein at a dose level of about 0.3 mg CN^- and about 0.8 mg SCN^- per kg body weight. These doses gave no visible toxic symptoms. The animals were killed at various intervals by immersion in a mixture of solid carbon dioxide and acetone (— 78° C). One mouse of each sex was sacrificed at 5 min, 20 min, 1 hour, 4 hours and 24 hours after the injection.

Autoradiography

The animals were sectioned and autoradiograms prepared according to the method for autoradiography of small laboratory animals developed by ULLBERG (1954, 1958). Sagittal sections, 20 μ thick, through the whole animals were made at different levels. The sections were dehydrated at — 10° C. The film used was Gaevert Dentus Rapid, and the exposure time was 195 days for the $KC^{14}N$ and about 10 days for the $KS^{35}CN$ experiments.

Attempts were made to prevent the escape of hydrocyanic acid from the sections by different methods (exposure at very low temperatures, enclosure of section + film in sealed plastic bags during exposure or treatment of the fresh sections with 1 per cent $AgNO_3$). These procedures, however, did not appear to affect the autoradiographic pictures.

Chemical identification of thiocyanate

The secretion of thiocyanate in the gastric juice after cyanide injection was studied in the following manner. Mice were kept on a synthetic diet (FROMAGEOT and ROYER 1943) for 5 days in order to reduce the content of exogeneous thiocyanate in the stomach. The animals then received 0.01 ml per g body weight of 0.002 M KCN (corresponding to a dose of 0.05 mg HCN per kg body weight; LD_{50} about 0.7 mg HCN per kg body weight) by intravenous injection into a tail vein. The animals were killed 10 min later and the stomach removed, opened, and washed with 7.0 ml of distilled water. The combined gastric contents and washings were then deproteinized by the addition of 0.5 ml of 10 per cent $Zn\ SO_4 \cdot 7\ H_2O$ and 2.5 ml of 0.1 n NaOH and centrifuged whereupon the supernatant was analyzed for thiocyanate according to NYSTRÖM and SÖRBO (1957).

Results

Since the autoradiographic patterns obtained for $KC^{14}N$ appeared to be almost identical with those with $KS^{35}CN$, the autoradiograms will be described without any distinction being made between them.

The overall distribution pictures from animals killed at various intervals after the injection differed very little, the final distribution pattern being largely established by as early as after 5 min.

The concentration of isotope in the blood is higher than in most tissues and persisted at this level during the period studied. In all autoradiograms the

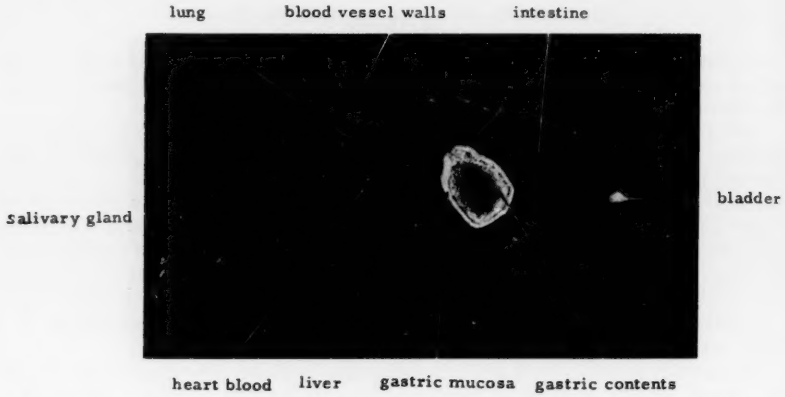

Fig. 1. Autoradiogram showing the distribution of C^{14} in a mouse 5 minutes after intravenous injection of $KC^{14}N$. Note high concentration in the gastric mucosa and the gastric contents close to the mucosa and in the bladder. No accumulation can be seen in the intestine.

highest activity is seen in the gastric mucosa and contents while the mucosa of the small intestine does not show any sign of accumulation and excretion. A fairly high concentration of radioactivity also appears in the salivary glands. A higher activity than in the blood is also found in the bladder and the walls of the large arteries. An accumulation can also be seen in cartilages and tendons. In the rest of the body the activity is much lower, and the central nervous system shows hardly any activity.

A detailed study of the autoradiograms reveals the following facts. A very high concentration can be seen in the gastric mucosa and in the gastric contents adjacent to the mucosa by 5 min after injection (Fig. 1). The secretion of the isotope is limited to the ventral part of the stomach whereas no significant activity is found in the nonsecretory oesophageal part. Later on, the whole gastric lumen contains a very high amount of radioactivity.

Since the highest activity was found in the stomach in both the cyanide and thiocyanate injected animals, it was concluded that the labelled compound secreted there was in both cases thiocyanate. That animals injected with cyanide secrete a significant part thereof as thiocyanate in the stomach was demonstrated by chemical analysis. Thus the gastric contents from 6 animals injected with cyanide contained 53.2 ± 15.6 mμ mol CNS^- as compared with the average value 13.0 ± 8.7 mμ mol of the control animals.

The small intestine and its contents shows a very low activity indicating a rapid reabsorption of the thiocyanate secreted in the stomach. The activity in the mucosa of the large intestine, on the other hand, is relatively high (Fig. 2).

An activity almost as high as in the stomach is found in the submaxillary

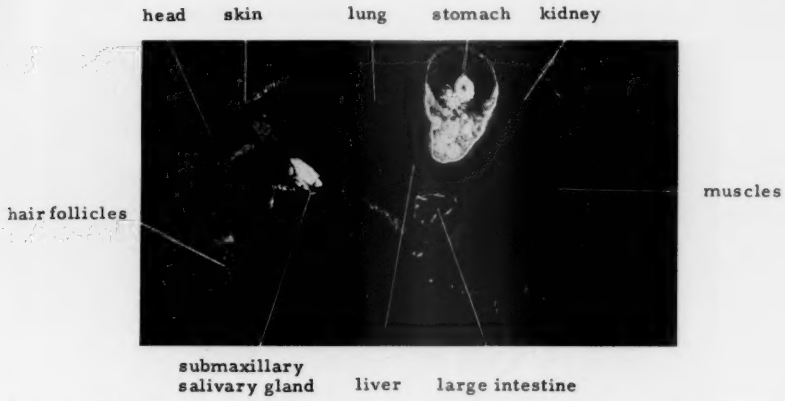

Fig. 2. Autoradiogram showing the distribution of S^{35} in a mouse 24 hours after injection of $KS^{35}CN$. White areas in the figure correspond to high S^{35} content.

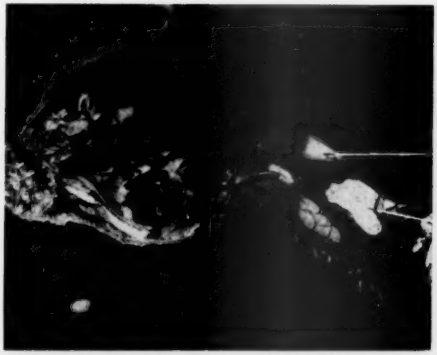

Fig. 3. Autoradiogram showing the distribution of S^{35} in the head and neck region of a mouse 4 hours after intravenous injection of $KS^{35}CN$. The concentration in the thyroid is somewhat higher than in the blood.

salivary gland whereas the sublingual gland contains much less of the isotope. Regarding the parotid gland, which was not present in our autoradiographic material, LOGOTHETOPOULUS and MYANT (1956 a) have already shown an accumulation of thiocyanate in this organ. The thyroid gland has an isotope concentration significantly higher than that of the blood and of most surrounding tissues (Fig. 3). The walls of the large arteries contain a very high activity, considerably higher than that in the blood. This is especially true for the $KC^{14}N$ injected animals.

Concerning the lymphatic tissues, it can be stated that the activity in the lymph nodes and in the thymus is low. Thus, the lymph follicles of the spleen

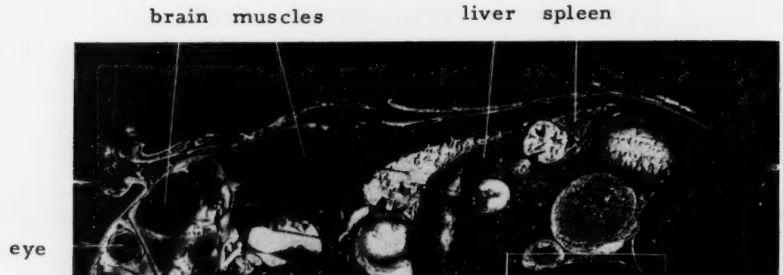

Fig. 4 a. Autoradiogram showing the distribution of S^{35} in a pregnant mouse 1 hour after intravenous injection of $KS^{35}CN$.

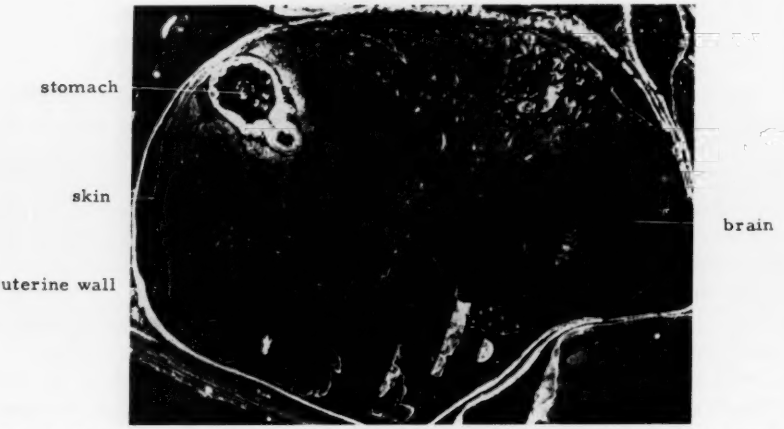

Fig. 4 b. Enlargement of detail from fig. 4 a showing high uptake of radioactivity in the gastric mucosa of a foetus.

have a lower concentration than the red pulp and the cortex. The activity of the lymph in the thoracic duct is significantly lower than in the blood.

In the skeleton, the highest activity is seen in the epiphyseal cartilages whereas the compact bone is less active, and the activity in the bone marrow is very low. Both hyaline and elastic cartilage have a rather high activity. Some accumulation can be found in the teeth and the highest concentration

appears to be located in the cementum although some activity is also found in the dentin. The activity is fairly high in the skin, especially in the hair follicles and in the basal part of the hairs.

The skeletal muscles, in general, show a fairly low concentration but in the tendons the activity is somewhat higher. The diaphragm also has a higher radioactivity in the connective tissue parts than in the muscular parts. The activity in the myocardium is slightly higher than in the skeletal muscles.

In the eye, some activity is present in the peripheral part of the lens and in the iris, cornea and sclera. No activity is seen in the aqueous humor.

The central nervous system has the lowest activity of all the tissues examined. This can be attributed to the fact that thiocyanate does not permeate the blood-brain barrier (*cf.* FRIEDEMANN 1942, STØA 1957). On the other hand, the autoradiograms of pregnant mice (Fig. 4) show that thiocyanate readily crosses the placental barrier. It is noteworthy that the foetal membranes, especially the chorion-allantois, take up activity. The full-term foetuses concentrate the isotopes in their gastric mucosa.

Discussion

The *in vivo* conversion of cyanide to thiocyanate has been known for a long time (LANG 1933). As previously stated, available evidence supports the assumption that the radioactivity in the sections from the cyanide injected animals is due to thiocyanate. The question then arises whether the activity in the thiocyanate autoradiograms in due solely to intact thiocyanate or to sulfur-containing metabolic products. It is known that thiocyanate is slowly oxidized to sulfate (WOOD, WILLIAMS and KINGSLAND 1947). The fact that the S^{35} concentration in the cartilage and foetal bones is not significantly higher than the C^{14} concentration in the same tissues indicates that the conversion of thiocyanate to sulfate is not important in this connection.

The rapid secretion of thiocyanate in the stomach deserves special attention. As early as 1877, GSCHEIDLEN observed that gastric juice contains thiocyanate but different opinions have been expressed concerning its origin. STUBER and LANG (1934) claimed that the thiocyanate was mainly due to swallowed saliva but this opinion was disputed by others (RIECKE 1933, GABRIELI 1950). LOGOTHETOPOULUS and MYANT (1956 a) clearly demonstrated that an active secretion of thiocyanate takes place in the surface epithelium of the gastric mucosa. This has been verified by the present work.

That thiocyanate is concentrated in the salivary glands is not unexpected as thiocyanate is a well-known constituent of normal saliva (LICKINT 1924, STØA 1957). It is surprising, however, that the sublingual, unlike the submaxillary (present work) and parotid glands (LOGOTHETOPOULUS and MYANT 1956), did not take up any thiocyanate. This may be related to the different secretion of the different salivary glands (see DUKES 1947).

The present work demonstrates (see Fig. 3) that the thyroid gland concentrates thiocyanate to some extent, which is in agreement with the autoradiographic results of LOGOTHETOPOULUS and MYANT (1956 b) who found the thiocyanate to be concentrated in the colloid. This, however, appears to be in disagreement with the results of WOOD and WILLIAMS (1949) and WOLLMAN, REID and REED (1958), who found the $S^{35}CN$ content of thyroid tissue to be less than that of blood. As explained by LOGOTHETOPOULUS and MYANT (1956 b) this is due to the fact that the colloid represents only a rather small fraction of the tissue.

A peculiar finding was the concentration of $S^{35}CN^-$ and C^{14} from cyanide in the walls of the large vessels. Whether this reflects any special metabolic activity of this tissue is not known but further studies of this phenomenon in various normal and pathological conditions are planned.

It is evident from the present work that thiocyanate is unequally distributed in the body and concentrated in certain organs, especially in the stomach. This may introduce serious errors in the determination of extracellular volume with thiocyanate (thiocyanate space) (EDER 1951) as previously indicated by others (KALTREIDER *et al.* 1941, FISHMAN and FISHMAN 1948). This may apply especially to cases of achylia and gastric resection.

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The Removal of Artificial Fat Emulsions from the Blood Stream of Dogs

By

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Abstract

Edgren, B. *The removal of artificial fat emulsions from the blood stream of dogs.* Acta physiol. scand. 1960. *48.* 390—401. — Cottonseed-oil emulsions were given intravenously to dogs in different doses and the disappearance of the injected fat from the blood stream was followed turbidimetrically. It was found that the fat was removed from the circulating blood with a speed that followed a single exponential function and that the disappearance rate decreased with increasing doses, if the rate of disappearance was expressed by the slope of the exponential regression line (K-value) or by the percentage of removal per hour. The average intravascular half-life for 1.0 g of fat per kg is 20 min, and the maximal amount of emulsified fat that can be removed completely from the blood stream during a 24-hour period seems to be 2 to 3 g of fat per kg in the dog. Following a single 1-g-per-kg dose of fat, the ability to remove an equal dose is depressed and remains depressed for more than eight hours but returns to normal within 24 hours. Protamine depresses the elimination rate for intravenous fat with about 50 % during a limited period.

The aim of the present investigation was to study the dynamics of the elimination of emulsified fat from the blood stream with special reference to the relation between the administered dose of fat and the elimination rate. Numerous authors have studied the disappearance of emulsified fat from the circulating blood in animals (Bloor 1914, Rony and Mortimer 1931, Waddell *et al.* 1953 a) as well as in normal subjects and patients (Johnson, Freeman and Meyer 1952, Mueller 1957, Kauste 1958), but in those investigations the relation between the rate of removal from the blood stream and the injected dose was not studied.

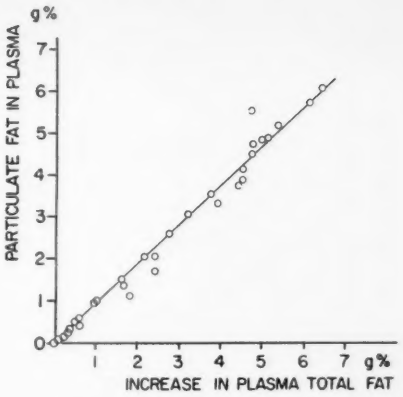

Fig. 1. *Comparison between gravimetric and turbidimetric determination of fat in plasma after intravenous fat emulsion in dogs.* Abscissa: plasma total fat, reduced by the plasma-total-fat value before starting fat infusion. Ordinate: emulsified fat in plasma. The data were compiled from five fat infusions with simultaneous determination of plasma total fat and emulsified (particulate) fat in plasma. The administered doses ranged from 1.0 to 6.6 g per kg.

Since the author has been able to show (EDGREN 1960 a, b) that post-heparin-clearing factor plays a role in the uptake of emulsified fat from the blood stream, the ability of protamine to delay the disappearance of fat *in vivo* was also investigated.

In a preliminary study (EDGREN 1957) was found that the rate of disappearance of emulsified fat decreased significantly with increasing doses, which suggested that the elimination pathways were partially blocked after the uptake of a given amount of fat. This observation prompted me to make experiments with repeated doses of fat in order to investigate this block further.

Materials and methods

Fat emulsion. A 25 % cottonseed-oil emulsion, stabilized with purified soybean phosphatides and Pluronic was used in all the experiments; its composition and preparation has been described in detail in an earlier publication (EDGREN 1958).

Determination of emulsified fat. The amount of emulsified fat was determined turbidimetrically by the method of GEYER, MANN and STARE (1948), by which the concentration of emulsified fat can be determined in plasma as well as in whole blood. In the original description of the method it was stated that any blood pigments disturbing the optical density readings should be eliminated by treating the samples with H_2O_2, and that aggregates of clustered fat particles should be dispersed with ammonia. Since no significantly disturbing haemolysis was observed and no aggregates could be seen in plasma microscopically, these procedures were omitted. The optical density was read in a Hilger Biochem colorimeter at 700 mμ using 13 mm round cuvettes. The samples were diluted with isotonic glucose solution to such an extent that the optical density readings lay below 0.60 extinction units. To confirm the accuracy of the method, it was in some experiments compared with BRUN's (1939) gravimetric method for determination of total fat in plasma. The result of these experiments, shown in Fig. 1, confirmed the reliability of the turbidimetric method within a wide range of levels of emulsified fat. Duplicate determinations were made with both the gravimetric and the turbidimetric method. In most of the experiments the fat level was determined in citrated

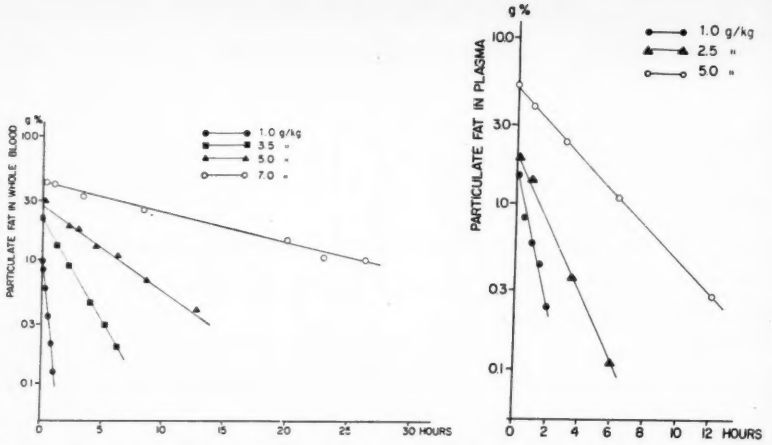

Fig. 2. *Removal of different doses of fat emulsion from the blood stream of dogs. Left fig.:* Removal of 7.0, 5.0, 3.5 and 1.0 g of fat per kg. The level of emulsified fat was followed in whole blood. *Right fig.:* Removal of 5.0, 2.5 and 1.0 g of fat per kg from plasma. The level of emulsified fat was followed from the end of the infusions. All the doses greater than 1 g per kg were administered as infusion, while 1 g per kg was given as a single injection with syringe.

plasma. In experiments where frequent sampling was necessary, however, the determinations were made in whole blood, since such determinations required smaller volumes of blood.

The error of the method for determination of emulsified fat in whole blood and plasma was determined by the method used in a previous paper (EDGREN 1960 a). When determining emulsified fat in plasma, the error in a duplicate was $\pm$ 3.5 % as calculated from 34 duplicates. The error in a duplicate determination of emulsified fat in whole blood, calculated from 30 duplicates, was $\pm$ 3.8 %.

Dog experiments. Adult non-fasting and non-anaesthetized dogs were used. The different doses of fat given were always calculated per kg body-weight. The 1-g-per-kg doses were given as single intravenous injections with syringe and needle intravenously during $1^1/_2$ to 2 min. The larger doses were given as intravenous infusions, where the drip rate was 10 ml of emulsion per kg body-weight and hour (2.5 g of fat per kg and hour).

1. *Experiments with different doses of fat.* Fat emulsion was given in doses ranging from 1.0 to 7.5 g per kg, and the disappearance of the fat was followed by analysis of the level of emulsified fat in plasma or whole blood at different times. A typical set of experiments are shown in Fig. 2. Since the disappearance of the infused fat from the blood almost invariably showed a single exponential regression, the over-all rate of elimination in each experiment could be expressed by the *K-value*, calculated from the formula of NEVEU *et al.* (1956).

$$K = (\log C_1 - \log C_2) / (T_2 - T_1) \qquad (1)$$

where C_1 is the blood level at the time T_1 and C_2 the blood level at the time T_2 on the disappearance curve $(T_2 - T_1)$ time units later. In the actual calculations time was expressed in hours.

The exponential disappearance of a substance from the blood stream implies that a

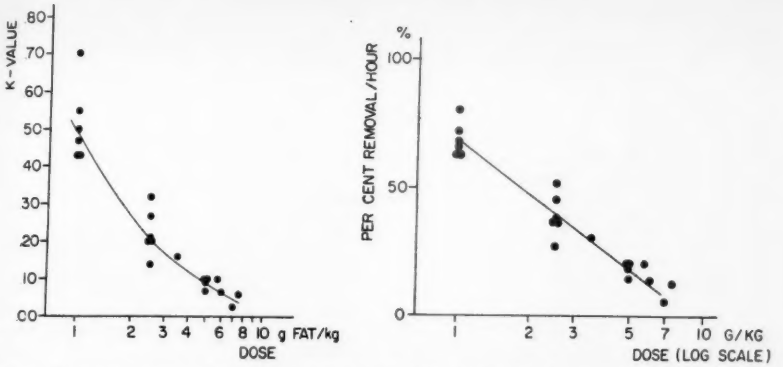

Fig. 3. a) *Relation between K-value and injected dose of fat.* b) *Relation between injected dose of fat and percentage of removal per hour.* The methods for calculating K-value and percentage of removal per hour are described under Materials and methods.

constant percentage of the circulating substance is removed per time unit, and that the absolute amount removed per time unit is largest initially, to decrease successively with time. The percentage of the circulating amount of fat removed per hour is related to the *K*-value according to the formula:

$$\text{per cent removal per hour} = 100 - 10^{2-K} \qquad (2)$$

The relation between the administered dose of fat and the *K*-value is shown in Fig. 3 a. and the relation between the dose and the percentage removal per hour is illustrated in Fig. 3 b.

2. *Evaluation of the 24-hour capacity for removal of fat from the circulation.* Fat emulsion was given to dogs in doses ranging from 1.0 to 5.0 g of fat per kg body-weight. The

Table I. Experiments to evaluate the 24-hour capacity in dogs to remove fat from the circulating blood

Dose of fat (g/kg)	Number of experiments	Number of dogs having turbid, slightly turbid, or clear plasma 24 hours after the start or infusion		
		Turbid plasma (>0.50 E)	Slightly turbid plasma (0.10—0.50 E)	Clear plasma (<0.10 E)
5.0	7	6	1	0
4.0	9	4	5	0
3.0	6	3	1	2
2.0	6	0	1	5
1.0	7	0	0	7

The optical density of citrate plasma was read at 700 mμ.

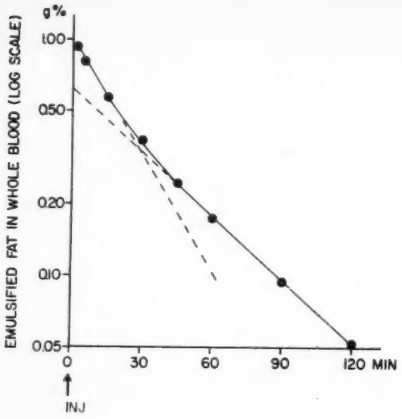

Fig. 4. *Average disappearance curve for 1.0 g of fat per kg from whole blood in dogs.* The curve represents the mean disappearance curve for six individual experiments. The average curve is composed of two exponential parts, an initial rapid phase and a slower phase.

turbidity of plasma was then determined exactly 24 hours after the start of the infusion. The dogs were fasting during this 24-hour period. Plasmas with a turbidity at 700 mμ lower than 0.10 extinction units were considered as non-lipaemic, since this degree of turbidity seemed to be the upper limit for fasting untreated dogs. Plasmas with a turbidity between 0.10 and 0.50 E were denoted as "slightly turbid" and plasmas with a greater turbidity than 0.50 E as "turbid". The results of these experiments and the number of animals with each dose will be seen in Table I.

3. *The removal of single 1-g-per-kg doses.* 1 g of fat (4.0 ml 25 % fat emulsion) per kg body-weight was given intravenously as a rapid injection and the level of emulsified fat in whole blood was followed during 2 hours. In order to ensure accurate blood sampling, blood was drawn through an intravenous polyethylene catheter lying with the free end in the caval vein. The catheter was inserted into one of the superficial hind-leg veins before the experiments started and was kept free from clotting by a very slow saline drip.

4. *Experiments with repeated 1-g-per-hour doses.* In one series of experiments, three 1-g-per-kg doses were given at two-hour intervals, and the disappearance of the fat from the circulating blood was followed by estimation of the fat content in whole blood. The blood sampling was performed as described in detail in the above paragraph. As there usually was some emulsified fat left in the blood at the time of the second and third injection, the rate of disappearance was expressed by the time in minutes required for a fall in the initial concentration of emulsified fat in blood corresponding to 50 % of the first dose injected, *i. e.* the time necessary for the removal of a certain amount of circulating fat. A typical experiment is shown in Fig. 5, and the changes in the time factor on repeated administrations are set out in Table II.

In another type of experiments, two 1-g-per-kg doses were administered at intervals of 4, 8 and 24 hours. In these experiments the rate of elimination was expressed as the intravascular half-life of each injected dose. The result of these experiments is illustrated in Fig. 6.

5. *Experiments with protamine.* In five experiments, 2.5 g of fat per kg was infused during 1 hour in five dogs, and 5 mg of protamine sulphate[1] was injected immediately

[1] Protamin Vitrum.

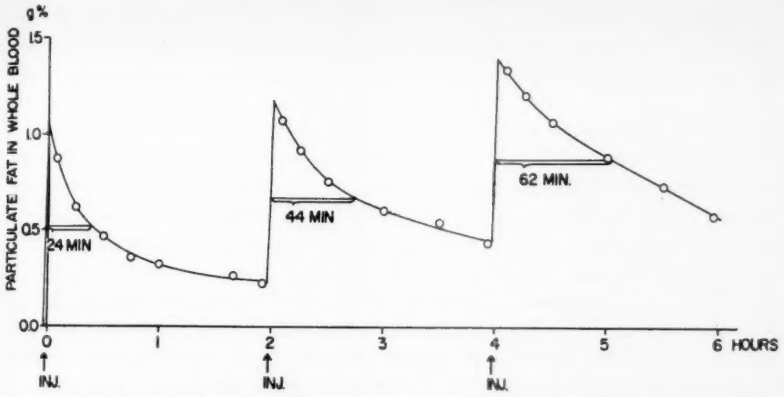

Fig. 5. *Removal from whole blood of three 1-g-per-kg doses, injected with 2-hour intervals.* As a measure of the elimination rate of each separate dose is taken the time necessary for a fall in blood-fat level corresponding to 50 % of the initial blood-fat level following the first injection. Since the injected doses of fat emulsion were not removed completely from the blood in the 2-hour intervals it was not suitable to use the intravascular half-life of the fat as a measure of elimination rate.

before, immediately after and 1 hour after infusion, and the fat level in plasma was followed for four hours after infusion. Seven control experiments were performed accordingly without protamine injections. The fall in the initial fat level during the first post-infusion hour expressed as a percentage of the initial plasma level of emulsified fat in the different experiments is given in the text to Fig. 7, where the average disappearance curves for 2.5 g of fat per kg with and without protamine injections are shown.

Results

The disappearance of varying doses of fat emulsion. It will be seen from Fig. 2 that the injected fat disappears exponentially from the blood stream, and that

Table II. The rate of disappearance of injected fat on repeated injections

	1st dose	2nd dose	3rd dose
Time in minutes for removal of an amount of fat corresponding to 50 % of the initial blood level after the first injection	22	36	64
	13	25	30
	24	44	62
	32	78	71
	12	15	34
	17	44	66
Mean and standard error (minutes)	20 ± 3.1	40 ± 8.9	54.5 ± 7.2

The mean time factor of the second and third injected doses differ significantly from that of the first dose.

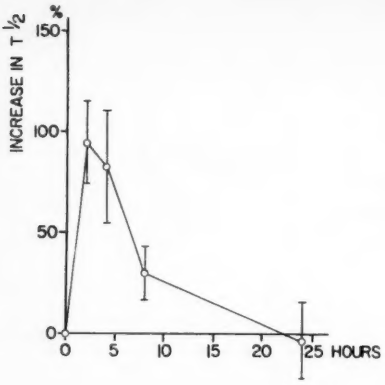

Fig. 6

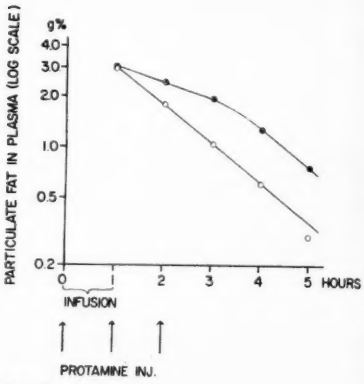

Fig. 7

Fig. 6. *The reduction of fat-eliminating capacity after a single 1-g-per-kg dose of fat emulsion.* Two 1-g-per-kg doses of fat emulsion were injected at different time-intervals: 2 hours (6 experiments), 4 hours (3 experiments), 8 hours (3 experiments), and 24 hours (4 experiments). Ordinate: percentage of increase of $T\,^1/_2$ (intravascular half-life) for the second dose as compared with the first dose in each experiment. Abscissa: time in hours between the injections. The points on the curve represent the mean results and their standard error.

Fig. 7. *Retardation of fat elimination by protamine.* Upper curve: mean disappearance curve of five infusions of 2.5 g of fat per kg with simultaneous protamine injections (5 mg of protamine injected three times as indicated by arrows). Lower curve: mean disappearance curve of seven infusions of 2.5 g of fat per kg without protamine. The average decrease in the plasma level of emulsified fat during the first post-infusion hour for the protamine group was 20.0 % of the initial blood concentration with a standard error of $\pm$ 2.84 %. The corresponding decrease for the control group was 40.3 $\pm$ 3.52 %. The difference between the two groups is statistically significant.

the slope of the regression line diminishes significantly with increasing doses. The consequence of this will be that a constant percentage of the circulating amount of fat is removed per time unit in each elimination process, and that this percentage decreases with increasing doses of fat. The percentage removed from the blood stream in 1 hour shows an inverse linearity with the logarithm of the administered dose of fat, as is illustrated in Fig. 3 b. The K-value shows a similar, though not linear, relationship with the injected dose (Fig. 3 a).

The 24-hour capacity for removal of artificial fat from blood. One consequence of the exponential removal of artificial fat from the circulating blood is that the level of emulsified fat in blood approaches the zero level extremely slowly, which makes it difficult to assess exactly after how many hours an injected amount is completely removed, especially after large doses. In order to study the 24-hour capacity, the turbidity of plasma was determined exactly 24 hours after the start of infusion of fat in doses ranging from 1.0 to 5.0 g per kg. The

results are given in Table I. It will be seen that 2 g of fat per kg and less is almost invariably eliminated completely from the blood within 24 hours, as is also, occasionally, 3 g of fat per kg. With 4 g per kg and more, plasma is never completely freed from emulsified fat within 24 hours.

The disappearance of a single 1-g-per-kg dose. Fig. 4 shows the average disappearance curve, plotted on semilog paper, for six 1-g-per-kg doses of fat emulsion. It is evident that the disappearance curve for 1 g of fat per kg shows a more complex nature than do those for the larger doses, shown in Fig. 2. The disappearance curve was usually composed of two exponential fractions, the first of these accounting for about 50—60 % of the removal. The difference in slope is, however, small between the two exponential parts of the curve. The intravascular half-life of 1 g of fat per kg averaged in 6 experiments 19.6 min. with a standard error of $\pm$ 1.3 min.

The elimination of repeated 1-g-per-kg doses. The experiments with three repeated 1-g-per-kg doses at 2-hour intervals, the results of which are given in Fig. 5 and Table II, showed that the rate of elimination decreases considerably with repeated injections. The changes in the time factor (the time in minutes required for a fall in the blood-fat level of 50 % of the initial blood concentration of the first dose) with repeated doses are statistically significant.

From the experiments illustrated in Fig. 6 it will be seen that the capacity to remove fat from the circulating blood is depressed for many hours after a preceding dose of fat has been completely eliminated, and that this depression of the elimination rate still remains eight hours after a "first" injection. The ability to eliminate fat has returned to normal after 24 hours.

The effect of protamine injection on the elimination rate. Intravenous injection of 15 mg of protamine sulphate, divided into three injections, delayed the elimination from the blood of 2.5 g of fat per kg during the first post-infusion hour by about 50 % in comparison with the controls, as is illustrated in Fig. 7. The slowing of the disappearance rate was statistically significant.

Discussion

The observation that the removal of emulsified fat from the circulating blood of dogs follows a single exponential regression, at least during the major part of the removal, has been reported earlier in a preliminary communication (EDGREN 1957) and is consistent with KAUSTE's (1958) observations in children and with the investigation of WADDELL *et al.* (1953 a) in rats. The exponential regression in the blood level of emulsified fat implies that the absolute amount of fat removed per time unit is largest initially and decreases with falling blood concentration, a fact that has also been pointed out by JOHNSON, FREEMAN and MEYER (1952).

From the present experiments it is apparent that emulsified fat is removed from the circulating blood of dogs at a rate that is inversely proportional to

the administered dose, if the rate is either calculated as a percentage of the circulating amount removed per hour, or expressed by the slope of the exponential regression line (K-value). This observed relation between the dose given and the elimination rate agrees with the observations by FRENCH and MORRIS (1957) on the removal of dietary ^{14}C-labeled chylomicra from the circulating blood in rats, although the injected doses in their experiments were considerably smaller (about 0.05 to 0.4 g per kg) than those administered in the present investigation, where the doses ranged from 1.0 to 7.5 g per kg. The elimination of fat emulsion and chylomicra has many features in common with the behaviour of injected colloids such as for instance carbon particles (BIOZZI *et al.* 1953). One lipoid that seems to be removed from the blood in a similar way is cholesterol. FRIEDMAN, BYERS and ROSENMAN (1954) demonstrated the essential role of the reticulo-endothelial system in the uptake from the circulation of dietary cholesterol in rats and NEVEU *et al.* (1956) showed that the removal of rabbit plasma cholesterol injected into rats followed an exponential function, and that the rate of removal was inversely proportional to the injected dose. Numerous workers have observed an accumulation of fat-stainable material in the reticulo-endothelial cells of the liver, spleen and lungs after intravenous injection of fat emulsion (SAXL and DONATH 1926, KIMURA 1937 a, b, WOERNER 1949, ASADA 1954). According to ASADA's (1954) interpretation of such findings the RES-cells of the liver would play the role of important mediator cells, taking up artificial fat particles from the blood by phagocytosis and delivering them to the neighbouring parenchymal cells of the liver. WADDELL *et al.* (1954), on the other hand, were not able to demonstrate any slowing of the uptake of fat emulsion from the circulating blood in rabbits by substances known to block the phagocytosis by RES-cells, despite the fact that these RES-blocking substances prevented the fat droplets from being disposed in the reticulo-endothelial cells. It therefore seems plausible that a small proportion of the injected artificial fat particles are treated as foreign bodies by the organism and are ingested by and concentrated in RES-cells, while the rest of the particles follow similar pathways as normal chylomicra, which are not concentrated in the reticulo-endothelial system (MURRAY and FREEMAN 1951). This view is also held by BEVILACQUA *et al.* (1954), who studied the morphological distribution of injected fat emulsion in dogs. Even if fat particles can be metabolized to a small extent in Kupffer's cells of the liver (SENO 1955) it seems as if the metabolic rate is low compared with that in the parenchymal cells of the liver. The deposition of fat particles in the RES-cells after intravenous administration of fat might therefore give an exaggerated picture of the role played by the reticulo-endothelial system in the removal of artificial fat from the blood stream.

The observation that the rate of disappearance decreased with increasing dose of injected fat suggested that the eliminating mechanism would be blocked or saturated. The experiments with repeated 1-g-doses clearly showed that

after one fat injection there is a "refractory" state persisting for many hours, during which a new dose is eliminated at a slower rate than is the first injected dose. This finding is at present difficult to explain. As suggested above, one explanation might be that the eliminating pathways are blocked or saturated in one way or other. This saturation might occur in the RES-cells, but, as discussed above, the reticulo-endothelial system seems to be of minor importance in the elimination of artificial fat from the blood stream. Since artificial fat is taken up from the blood mainly by the liver (BEVILACQUA *et al.* 1954, WADDELL *et al.* 1953 b), which is also the principal organ for the uptake of chylomicron fat (FRENCH and MORRIS 1958, BORGSTRÖM and JORDAN 1959), it seems more likely that the saturation effect occurs in the liver cells. Although massive doses of artificial fat administered daily will cause liver damage in dogs (EDGREN 1960 c) it seems unlikely that a dose of fat as small as 1 g per kg would produce a depression in liver function and hence be the cause of the observed lowering of the ability to remove fat.

The 24-hour capacity in normal dogs to eliminate artificial fat from the circulating blood was 2—3 g of fat per kg. This finding agrees with the observation that 3 g of fat per kg and day seems to be the largest amount to be well tolerated by dogs in chronic experiments (EDGREN 1960 c). Thus, it seems probable that daily infusion of more fat than can be eliminated during 24 hours might have toxic effects on the body.

Since it has earlier been shown (EDGREN 1960 b), that intravascular lipolysis probably accounts for part of the removal of artificial fat from the circulating blood in dogs and that the ferment being responsible for the main part of this lipolysis seems to be postheparin-clearing factor, the experiments with protamine were carried out in order to quantitate the participation of intravascular lipolysis in the uptake of emulsified fat from the blood (Fig. 7). It was found that protamine delayed the elimination during the first post-infusion hour by about 50 % as compared with untreated animals. The effect of the injected protamine lasted for about 2 hours after the last injection, the disappearance rate thereafter approaching the rate in the control animals. The difference in maximal blood concentration after completed infusion between normal and protamine-treated animals is small, which indicates that protamine does not delay the elimination to any great degree during infusion, the effect being most apparent during the first two post-infusion hours. This observation is consistent with the observation (EDGREN 1960 b) that the lipolytic activity in dog plasma after intravenous fat is maximal 1—2 hours after infusion. Protamine and other antiheparin agents inhibit the clearing factor *in vitro* (KORN 1955) and diminish the production of clearing factor after heparin injection. Protamine also diminishes the rate of elimination of fat emulsion (MOELLER *et al.* 1956) and of injected fatty chyle (FRENCH and MORRIS 1957) from the blood and increases the postprandial lipaemia in man (DAY *et al.* 1957), observations indicating that postheparin-clearing factor in some way is concerned in

the transport of both chylomicron fat and fat emulsion from the circulating blood to the organs. The present investigation suggests that during a certain period of the elimination process clearing factor accounts for a considerable part of the removal of the injected fat, possibly as much as 50 % of the elimination. But the possibility that protamine may have some other effects in addition to that of inhibiting the action of clearing factor and circulating heparin and heparinoids, cannot be ruled out, since protamine has a tendency to "clump" fat emulsions, at least *in vitro* (MOELLER *et al.* 1956). After protamine injection, however, considerable elimination still took place, which indicated that the greater part of the uptake of the fat particles from the blood occurs without the intervention of clearing factor, and that this part is taken up as intact particles from the circulating blood.

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Acta physiol. scand. 1960. 48. 402—405

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The Morphological Distribution in the Mouse Liver of Injected Paraffin Emulsion

By

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Received 27 August 1959

Abstract

Edgren, B. and B. Ivemark. *The morphological distribution in the mouse liver of injected paraffin emulsion.* Acta physiol. scand. 1960. *48.* 402—405. Liquid paraffin was injected intravenously into mice in the form of an emulsion. The animals were killed 24 hours after injection and their livers investigated histologically. As controls were used one group of mice in which the emulsifiers had been injected and one group of untreated animals. It was found that in most of the paraffin-treated mice lipid-stainable droplets were present both in Kupffer's cells and the parenchymal cells of the liver far more abundantly than in the controls. The findings indicate that finely, emulsified liquid paraffin can be taken up by the parenchymal cells of the liver from the circulating blood.

During recent years the mechanism by which dietary chylomicra and emulsified artificial fat is taken up from the blood stream into the organs has been the subject of special interest. It has been demonstrated in earlier publications that intravascular lipolysis, presumably by the action of clearing factor, accounts for part of the removal of artificial fat emulsion from the circulating blood in dogs (Edgren 1960), but the experiments indicated that a great part of the fat was probably taken up from the blood by a non-hydrolytic mechanism, possibly as intact particles. Such a mechanism has also been proposed for the uptake of normal chylomicra from the circulating blood (French and Morris 1957).

In an earlier publication (Edgren and Wretlind 1958) it was shown that emulsions of liquid paraffin, a hydrocarbon whose physical properties resemble those of the lipids especially with regard to solubility, and which cannot be

Table I. Composition of Injected Emulsions

	Paraffin emulsion	Emulsifying-agent emulsion
Liquid paraffin[1]	25 g	—
Purified soybean phosphatides[2]	1.2 g	1.2 g
Demal 14[3]	0.5 g	0.5 g
Isotonic glucose ad	100 ml	100 ml

[1] Paraffinum liquidum, according to the standards of the Swedish Pharmacopoeia.

[2] The crude soybean phosphatides (from Glidden Co., Chicago, Ill., U. S. A.) were purified by the method described by WRETLIND (1957).

[3] Demal 14 = polyglycerol monooleate. From Emulsol Corp., Chicago, Ill., U. S. A.

hydrolyzed in the circulating blood, was removed from the blood stream in rabbits. It was therefore considered of interest to find out whether particles of paraffin could enter the liver cells in mice.

Experimental

Emulsions. The emulsions employed had the composition given in Table I and were prepared by high pressure homogenisation, by the procedure described in detail in a previous paper (EDGREN 1958). The particle size of the paraffin emulsion averaged about 0.5 μ.

Animal experiments. Adult white laboratory mice of both sexes with a body-weight ranging from 26 to 35 g were used. Paraffin emulsion was injected into the tail vein in 11 animals in an amount of 1.0 ml per 100 g body-weight (2.5 g of liquid paraffin per kg). The same amount of emulsifying-agent emulsion was injected in 10 mice which served as controls, and as a second control group were used 5 untreated mice. The animals were killed 24 hours after injection for histological examination.

Histological technique. The livers, and in some cases the lungs, were dissected out and fixed in 10 % formalin. Frozen sections with a thickness of 10 μ were stained with Sudan III, and in addition sections of paraffin-embedded tissue stained with hematoxylin-eosin were prepared. The amount of fat-stained material in the parenchymal cells was graded from — to +++, and the result of the semi-quantitative evaluation is shown in Table II. All the sections were mixed at random before examination and the procedure was repeated twice, giving mainly the same distribution of results.

Results

Table 2 shows the results from the microscopic evaluation of the occurrence of fat-stainable material in the parenchymal cells of the liver. It will be seen that the animals in the paraffin group differed from those in the emulsifier group and from the untreated group with regard to the amount of fat-stainable material in the parenchymal cells. The emulsifier group and the untreated group differed only slightly from each other. Fat-stainable droplets were present also in Kupffer's cells of the paraffin-treated animals. From each of the experimental groups frozen sections of the lung were stained for fat in some

Table II. Degree of Occurrence of Lipid-Stainable Material in Mouse-Liver Parenchymal Cells after Intravenous Paraffin Emulsions

	Total number of animals	Number of mice showing a certain amount of fat-stainable material in parenchymal cells			
		–	+	++	+++
Paraffin group	11	0	2	5	4
Emulsifier group	10	6	4	0	0
Non-treated group	5	3	2	0	0

For further explanation, see the text.

animals, and it was found that in the paraffin-treated animals small amounts of fat-stained particles were present in alveolar macrophages, while no such finding was made in the lungs from mice in the two control groups.

Discussion

From the actual investigation it is evident that intravenous injection of emulsified liquid paraffin into mice increases the amount of lipid-stainable material both in Kupffer's cells and the parenchymal cells of the liver as seen 24 hours after injection. The fact that the paraffin-treated animals differed in this respect from the animals treated with emulsifiers only (soybean phosphatides and polyglycerol monooleate) indicates that the fat-stainable material observed histologically consisted mainly of liquid paraffin, which in one way or other had been taken up by the parenchymal cells.

As was expected, some of the injected emulsified liquid paraffin was found in Kupffer's cells and in alveolar macrophages, which indicated a removal from the blood by phagocytosis. In this respect the emulsified liquid paraffin resembles artificial fat emulsion, which is also taken up by reticulo-endothelial cells in the body when injected intravenously (SAXL and DONATH 1926, WOERNER 1949, BEVILACQUA *et al.* 1954, ASADA 1954). In the case of artificial fat emulsion, however, the quantitative role played by the R. E.-cells in the removal of fat from the blood stream seems to be of minor importance (WADDELL *et al.* 1954), and normal chylomicra do not seem to accumulate at all in Kupffer's cells or other R. E.-cells (MURRAY and FREEMAN 1951). It is probable that the reticulo-endothelial cells in the case of emulsified paraffin account for part of the uptake of the particles from the blood.

ASADA (1954) and SENO (1955) have proposed a mechanism for the removal of artificial fat emulsions from the blood by the liver, by which mechanism the fat particles are conveyed to the parenchymal cells of the liver by the Kupffer cells, which have taken up the fat particles from the circulating blood by phagocytosis. In the present investigation it was found that the amount

of fat-stained material increased significantly in the parenchymal cells of the livers of mice that had been injected with emulsified liquid paraffin, observations that indicate that paraffin particles can enter the parenchymal cells of the liver from the circulating blood. Whether the paraffin particles had been transported to the parenchymal cells by the Kupffer cells or penetrated from the blood into the parenchymal cells directly is difficult to establish from the acquired data, however.

The reported findings lend support to the hypothesis that particles such as dietary chylomicra and artificial fat emulsion may be taken up from the blood stream by the parenchymal cells of the liver, the most important organ for the removal from the blood of both chylomicra (FRENCH and MORRIS 1958) and artificial fat emulsion (WADDELL *et al.* 1953, BEVILACQUA *et al.* 1954), without primary intravascular lipolysis.

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Acta physiol. scand. 1960. 48. 406—416

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Muscle Training by Static, Concentric and Eccentric Contractions

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Abstract

BONDE PETERSEN, F. *Muscle training by static, concentric and eccentric contractions.* Acta physiol. scand. 1960. *48.* 406—416. — Isometric strength of the elbow flexors and the knee extensors was measured by a strain-gauge dynamometer constructed according to DARCUS (1953). The accuracy of this dynamometer was found to be ± 1—3 per cent. Seventeen young female and seventeen young male subjects underwent a training period of from 20 to 36 days. Their training constituted the performance of one of the following forms of exercise: 1 daily maximum isometric contraction, 10 daily maximum isometric contractions, 10 daily eccentric muscular contractions or 15 min of daily heavy dynamic work (riding a bicycle ergometer). The effect on the isometric strength and endurance of the trained muscle groups was measured and compared to a control group of 7 females and 6 males, who received no specific training. It was found that 1 daily maximum isometric contraction had no effect on the isometric strength of the muscles, 10 daily maximum isometric contractions had a tendency to increase the isometric strength of the muscles, while 10 daily maximum eccentric contractions had no measurable effect on isometric muscle strength. Heavy dynamic work increased the isometric strength of the muscles by 12 per cent in the females and 23 per cent in the males. There was no measurable effect on endurance to sustained isometric contractions consequent to any of the training programs. Training of muscles on one side did not modify the strength of the contralateral muscle groups.

HETTINGER and MÜLLER (1953) claimed that 1 isometric muscle contraction a day with a tension equivalent to 2/3 of the maximum strength of the muscle group and of 6 sec duration gave a maximum training response. However,

other workers have found that only strenuous exercises with many contractions would result in increased muscle strength (DELORME 1945, HELLEBRANDT and HOUTZ 1956). The experiments of HETTINGER and MÜLLER were performed on 9 subjects who trained in 71 different ways, mainly the muscles around the elbow joint. When the subjects trained 5 days a week, they found an increase in isometric strength of about 8 per cent per week.

In the present experiment it was decided to investigate the effect of 1 isometric maximum contraction daily on the isometric strength of muscles, as compared to the effect of 10 maximum isometric contractions per day and to the effect of heavy dynamic work. As it is possible during an eccentric muscular contraction to develop a tension which exceeds the maximum isometric tension (cf. ASMUSSEN 1952), a program of eccentric training contractions was also included, to investigate the effect of training by different tensions.

An endurance test was employed using the time a subject was able to maintain a certain load in a sustained contraction as a criterium of endurance. Muscle strength was measured by a dynamometer utilizing the torque principle first employed by BETHE and FRANKE (1919). DARCUS (1951, 1953, 1955) has developed a dynamometer operating on this same principle making use of modern electronic techniques. This dynamometer provides an objective measurement, and is easy to standardise.

HETTINGER and MÜLLER (1953) allowed the subjects to train on the dynamometer every day. This procedure could possibly modify the final results, since the subjects became familiar with the test method. In the present experiments the subjects trained on special devices and only used the dynamometer to measure muscle strength at specific intervals.

It has been claimed by HELLEBRANDT, PARRISH and HOUTZ (1947), DARCUS and SALTER (1955) and MATHEWS *et al.* (1956) that training of muscles on one side induces an effect on the contralateral muscle groups. The training in the present experiments was, therefore, unilateral for some muscle groups, while the testing was bilateral.

Methods and Procedure

Twenty-four adult females (f.) and 23 adult males (m.) participated in the experiments. None of the f. and one of the m. were over 30 years, none of the subjects were younger than 18. The training and testing programs are described below. The investigation was primarily concerned with the right and left elbow flexor muscles and the right and left knee extensor muscles. Preliminary measurements showed the standard deviation of the mean isometric strength on five different test days to be $\pm$ 4 per cent in a group of 4—5 subjects. It was, therefore, expected that the effect of training could be measured by the applied technique even if groups of only 4—5 subjects were trained, because, an increase of about 8 per cent per week should be expected as a result of training by only 1 daily isometric maximum muscle contraction (HETTINGER and MÜLLER 1953).

a) Test methods.

For measuring the isometric strength the DARCUS dynamometer was used. This

Fig. 1. Testing of the isometric strength (knee extensors) by the DARCUS dynamometer. Note that the axis of the knee joint is coincident with the axis of the dynamometer, which perforates the center of the dark disc also seen (cf. Fig. 2).

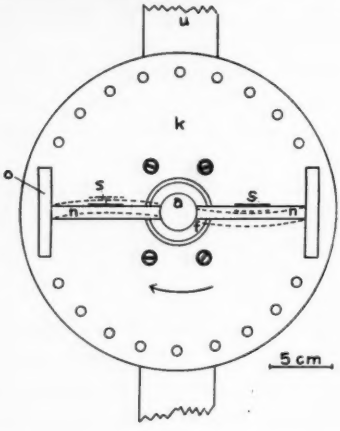

Fig. 2. The working principle of the DARCUS dynamometer (cf. Fig. 1). u = column supporting. k = vertical disc with holes. f = ball bearing. a = spindle. n = spring steel bar perforating 'a' . o = holder which fixes 'n'. s = strain-gauge (a resistance wire firmly adherent to 'n'). When 'a' is influenced by a torque as indicated by the arrow, 'n' is bended as shown by the dotted lines. It follows that the two strain-gauges to the left and right are respectively stretched and compressed, resulting in respectively an increase and a decrease in resistance. These variations in resistance are proportional to the torque, and were measured with a Wheatstone's bridge.

dynamometer measures the torque exerted about the axis of a joint. The principle of the dynamometer follows from Fig. 1—2. The limb whose muscles are to be investigated was fixed by a suitable holder to the spindle in such a way, that the axis for the attempted movement was coincident with the axis of the dynamometer. Only the part of the extremity distal to the joint to be tested was fixed by the holder, otherwise the subject was free.

The dynamometer was calibrated with a known torque and the error of the single measurement did not exceed $\pm$ 3 per cent.

A muscle test consisted of 5 consecutive trials. During each a maximum isometric contraction was performed. Each contraction lasted about 5 sec and was repeated at intervals of 1 min.

The DARCUS dynamometer was also used in the endurance test, where the maximum time the subject was able to hold 50 per cent of the maximum isometric torque was determined.

b) Training methods.

The *isometric training* was conducted as shown in Fig. 3 and 4. A strap was placed

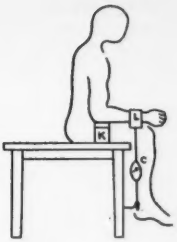

Fig. 3. Method of training the right elbow flexors by isometric contractions. C = COLLIN dynamometer for registering the pull. K = firm support. L = leather strap.

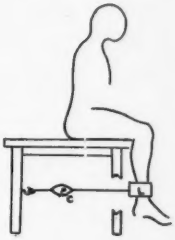

Fig. 4. Method for training the right knee extensors by isometric contractions. C = COLLIN dynamometer for registering the pull. L = leather strap.

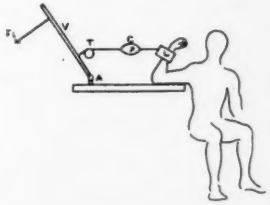

Fig. 5. Method of training the right elbow flexors by eccentric contractions. A = moveable joint. C = COLLIN dynanometer. Fl = arrow, which indicates the direction of the pull exerted by the experimental leader. V = lever arm. T = pulley. L = leather strap.

around the distal part of the extremity and the tension registered by a COLLIN's dynamometer, which could be used as a tensiometer. Pieces of thick felt were used as padding to enable the contractions to be carried out in comfort. Every contraction lasted about 5 sec and repeated contractions were made every 30 sec.

The method of training by *eccentric contractions* is illustrated in Fig. 5. A simple system of lever arms, a pulley and a wire allowed the elbow joint to be forced slowly into extension during 5 sec in spite of the maximum resistance of the subject. This was done every 30 sec and the joint was moved from 140°—45° of flexion. A COLLIN's dynamometer indicated the maximum tension developed. The maximum tension registered during this form of contraction, was about 20—30 per cent higher than that developed during the training by maximum isometric contractions.

The *dynamic training* was carried out on KROGH's bicycle ergometer. The work load employed was determined by the attainment of a heart rate of about 180 beats/min. The average work loads were, therefore, with 60 pedal revolutions/min, for the females 926—1,028 kgm/min and for the males 1,338—1,545 kgm/min.

c) Statistical methods.

The data were treated on the basis of paired samples, and the "null hypothesis" was rejected at the 0.02 level.

d) Training procedure.

Table I. Average isometric torque (maximum of five trials) in kg × cm of the trained right elbow flexors compared to the controls. The standard error is indicated

Sex	Time of Test	Group I 5 females 5 males 1 isom. contr/day	Group II 4 females 6 males 10 isom. contr/day	Group III 4 females 2 males 10 excentric contr/day	Controls 7 females 6 males no training
Females	before training	500 ± 70	460 ± 20	425 ± 32	469 ± 26
	after 9 days.....	452 ± 51	438 ± 37	385 ± 31	430 ± 29
	after 18 days.....	462 ± 63	448 ± 28	418 ± 18	431 ± 31
	after 27 days.....	452 ± 35	438 ± 13	435 ± 24	416 ± 38
	after 36 days.....	460 ± 36	465 ± 17	448 ± 23	433 ± 39
Males	before training	790 ± 48	829 ± 33	810 ± 32	820 ± 45
	after 9 days.....	810 ± 110	897 ± 35	855 ± 45	835 ± 47
	after 18 days.....	818 ± 56	860 ± 43	830 ± 60	782 ± 37
	after 27 days.....	826 ± 68	880 ± 57	840 ± 140	822 ± 49
	after 36 days.....	837 ± 78	962 ± 49	885 ± 49	839 ± 56

The subjects were divided into one control group and four training groups, so that the means of the body heights were practically equal.

Group I (5 f. and 5 m.) trained the right elbow flexors and the right knee extensors on 36 occasions within 60 days with *1 maximum isometric contraction per session.*

Group II (4 f. and 6 m.) trained the same muscles and in the same manner as group I but with *10 maximum isometric contractions per day.*

Group III (4 f. and 2 m.) trained the right elbow flexors with *10 eccentric contractions per day* on 36 occasions within 54 days.

Group IV (4 f. and 4 m.) trained as described above *15 min on* KROGH's *bicycle* on 20 occasions within 26 days.

The control group (7 f. and 6 m.) received no training, but was tested in the same manner as groups I—III.

e) Testing procedure.

In all of the groups the *isometric torque* of both the right and the left elbow flexors and knee extensors was tested before and after training. In addition the isometric torque of the right elbow and the right knee extensors were tested in groups I—III the day after the 9th, 18th and 27th day of training.

Strength endurance was measured before and after the training period, in the right elbow flexors and the right knee extensors of all groups, and in addition the endurance of the left knee extensors was measured in group IV.

The *strength of the finger flexors* was measured on both sides in all the groups before and after training. For this was used COLLIN's dynamometer.

Results

Tables I and II show that there was no increase in the isometric strength of the trained muscles, as a result of 1 maximum isometric contraction per day.

In group II who trained with 10 isometric contractions per day no significant increase was found in the two muscle groups trained. Expressing the increases

Table II. Average isometric torque (maximum of five trials) in kg × cm of the trained knee extensors compared to the controls. The standard error is indicated

Sex and Side	Time of Test	Group I 5 females 5 males 1 isom. contr/day	Group II 4 females 6 males 10 isom. contr/day	Group IV 4 females 4 males 15 min bicycle riding/day	Controls 7 females 6 males no training
Females					
right side	before training....	1,328 ± 141	1,128 ± 81	1,110 ± 45	1,319 ± 128
	after 9 days.....	1,206 ± 105	1,088 ± 46	—	1,224 ± 103
	after 18 days.....	1,210 ± 106	1,078 ± 76	—	1,229 ± 105
	after 20 days.....	—	—	1,278 ± 66	—
	after 27 days.....	1,228 ± 101	1,210 ± 91	—	1,246 ± 126
	after 36 days.....	1,230 ± 100	1,230 ± 87	—	1,233 ± 112
left side	before training....	—	—	1,140 ± 131	1,237 ± 110
	after 20 days.....	—	—	1.250 ± 122	1,224 ± 115
Males					
right side	before training....	1,964 ± 277	1,993 ± 131	1,670 ± 141	2,135 ± 129
	after 9 days.....	1,860 ± 264	1,993 ± 96	—	1,882 ± 76
	after 18 days.....	1,898 ± 204	1,945 ± 114	—	1,770 ± 69
	after 20 days.....	—	—	1,988 ± 150	—
	after 27 days.....	2,010 ± 269	2,141 ± 129	—	2,007 ± 121
	after 36 days.....	2,026 ± 230	2,215 ± 129	—	1,982 ± 119
left side	before training....	—	—	1,550 ± 101	2,225 ± 98
	after 20 days.....	—	—	1,956 ± 224	1,935 ± 150

as per cent change enables a statistical treatment of both the trained muscle groups as a single group. The increase in the female group was 5 per cent (not significant) and in the male 13 per cent with a significance of 0.01.

Group III, who trained with 10 eccentric contractions per day, failed to demonstrate any significant increase in the strength of the trained muscle group (Table I).

In group IV the training by dynamic work resulted in no significant increase in muscle strength, but when the knee extensors of both sides are treated as a single group an increase of 12 per cent in the females and of 23 per cent in the males with a significance of 0.01 was found. The difference between the increase in the males and in the females was not significant.

The control group showed no significant variations (Tables I—II).

Table III shows that there was no effect of 1 or 10 maximum isometric or 10 eccentric contractions daily on the contralateral isometric strength.

In none of the groups was an increase found in the strength of the finger flexors on the two sides. Nor was there any increase of the elbow flexors in group IV, who only trained the legs by dynamic work.

Table III. Variations in isometric torque (maximum of five trials) on the contralateral side during unilateral training compared to the controls

Muscle Group	Sex	Time of Test	Group I 5 females 5 males 1 isometric contr/day of right side	Group II 4 females 6 males 10 isom. contr/day of right side	Group III 4 females 2 males 10 eccentric contr/day of right side	Controls 7 females 6 males no training
left elbow flexors	females	before training of right side....	420 ± 41	413 ± 25	383 ± 25	449 ± 32
		after training of right side....	414 ± 32	368 ± 24	373 ± 25	416 ± 36
	males	before training of right side....	890 ± 96	813 ± 63	830 ± 30	817 ± 46
		after training of right side....	872 ± 89	817 ± 41	840 ± 50	788 ± 66
left knee extensors	females	before training of right side....	1,084 ± 103	1,140 ± 144	—	1,237 ± 110
		after training of right side....	1,261 ± 131	1,000 ± 105	—	1,224 ± 115
	males	before training of right side....	1,936 ± 291	1,887 ± 131	—	2,225 ± 98
		after training of right side....	1,954 ± 295	1,933 ± 144	—	1,935 ± 150

No systematic variations in muscle endurance could be demonstrated as a result of the training in any of the groups, possibly due to a large scattering of the results.

Discussion

The results of this study indicate that there was no effect of training by 1 isometric contraction per day, while training by hard dynamic work, although not of maximal intensity, increased the muscle strength. The effect of the training was in the present study limited specifically to the trained muscles. Other muscle groups did not benefit from the training.

Since these results are at variance with some recently reported results, an explanation of this discrepancy is necessary. HETTINGER and MÜLLER (1953) reported that a training program of 1 isometric contraction 5 days a week with a tension of 2/3 of the maximum, would induce an increase in isometric strength of 8 per cent per week. They found no greater effect if 1 maximum isometric contraction per day was performed. The program in the present experiments, essentially repeating HETTINGER and MÜLLER's work (1953) failed to confirm their findings. Although the number of training days averages

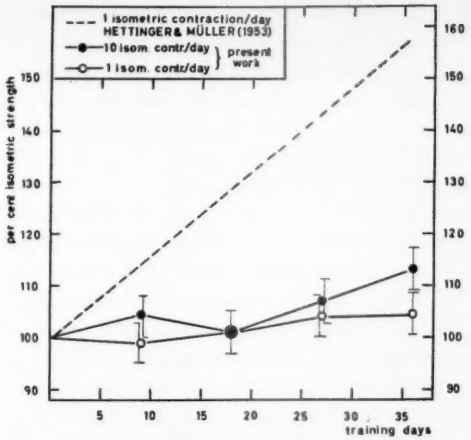

Fig. 6. Diagram showing the difference between the present results and the results of HETTINGER and MÜLLER (1953). Abscissa: number of days trained. Ordinate: isometric strength in per cent of its value before training. The single points are means of the strength of the elbow flexors and the knee extensors. Only the results from the male groups are brought.

only 4 per week in the present experiments, it would be unlikely that a certain amount of improvement would not have occurred. Increasing the number of contractions to 10 per day it was possible, by combining results of several muscle groups, to show an increase in strength in the male subjects of 13 per cent, *i. e.* one fifth of that expected from the results of HETTINGER and MÜLLER (1953) (Fig. 6). However, this increase could not be demonstrated in the female subjects.

DARCUS and SALTER (1955) have shown that individual muscle groups responded in different ways to the same form of training. It was, therefore, assumed that such statistical manipulations as combining results from several muscle groups was improper. Consequently, it could only be concluded, that a training program utilizing 1 or 10 maximum isometric contractions per day, was ineffective.

DARCUS and SALTER (1955) have also shown that 30 maximum isometric contractions per day in about 25 days did produce an increase in isometric strength of 18—46 per cent. The training contractions were of the duration as used in the present experiments.

SALTER (1955) found in a similar experiment increases of 52—88 per cent in isometric strength as a result of training by 30 maximum isometric contractions in 25 days. If these observations are correct, then it is possible that 30 maximum isometric contractions per day are apt to increase isometric strength, while one or even 10 maximum contractions per day are inadequate to induce changes. It would, therefore, be reasonable to assume that the isometric strength could increase by increasing the number of static contractions. The effect of

varying frequency of training contractions on the isometric strength cannot be evaluated from available data.

The influence of muscle tension during the training was also investigated in the present experiments by using eccentric contractions. In spite of the higher tension developed during this form of muscle contractions, no increase was found consequent to 10 daily eccentric contractions. No other experiments on eccentric training have been reported.

The influence of dynamic training on the functional capacity of muscles was investigated by DELORME (1945). He stressed the necessity of employing maximum contractions if increased muscular strength was a desired goal. This is also in disagreement with the present results. Here an increase in muscle strength was found after heavy work with a great number of submaximal contractions. However, in order to demonstrate a significant increase in strength as a result of this type of training it was again necessary to combine the results of symmetrical muscles. It was considered correct to do this, since the measurements showed, that there is very little difference between the muscles of the two sides. A slight tendency for a greater effect of both static and dynamic training was to be seen in the males than in the females, but the differences were not statistically significant.

DARCUS and SALTER (1955) found an increase in the strength of the contralateral muscles after unilateral training by 30 maximum isometric contractions daily. In the present experiments no such effect was found nor could any be expected, since there was no statistically significant increase in strength of the trained muscles. KRUSE and MATHEWS (1958) also failed to observe an increase in strength of the contralateral elbow flexors after a program of unilateral training, which was shown to increase the strength of the trained muscles. The reason for this disagreement is not understood.

DELORME (1945) claimed that if a muscle group should increase in endurance a training program of a greater number of submaximal dynamic contractions must be employed. No support for this general statement was found in the present experiments. The results showed that there was no measurable increase in endurance to sustained static contractions after training by submaximal dynamic contractions. It would be unlikely that the present dynamic training should not increase the endurance to dynamic work but unfortunately this was not tested. SAMSON (1953) showed that the result of an endurance test depends on the load, frequency and form of contractions used. Data from a dynamic endurance test could, therefore, not be compared to the results from the present static endurance test.

ASMUSSEN (1949) found a greater effect on endurance to static contractions after training by sustained isometric "work" than by intermittent isometric "work", but no significant effect on muscle power. He, therefore, suggested that it was possible to train the ability to perform anaërobic work without any demonstrable effect on sheer muscle power. No effect on the ability to

sustained isometric contractions was shown as a result of dynamic training in the present experiments. It is, therefore, suggested that the amount of static work performed during the training and not the number of contractions modifies the capacity to static "work" as claimed by DELORME (1945).

Until more exact information is available it must be stressed, that the generally accepted view, that endurance is best trained by many submaximal contractions, is founded upon insecure experimental evidence.

An important point which must be considered in evaluating the present as well as other results, relates to the instrumentation utilized to measure the increase in strength and endurance. If the same instrument is used for training and for testing, it is possible that improvement in skill would occur. This induces a factor which cannot be evaluated, nor is it clear if different test methods are subjected to this influence of skill in the same degree. The results of HETTINGER and MÜLLER (1953) and DARCUS and SALTER (1955) are both dependent upon an experiment where the same instruments are used for training and testing. The effect of the isometric training in these two works may, therefore, be attributed to improvement in skill. In the present experiments the influence of skill was eliminated by using different instruments for training and testing. The data from the control group confirmed the impression that the testing per se did not influence the results. The increases in the present experiments can only be attributed to a pure training effect.

According to DARCUS (1953) the method of testing the isometric torque excludes the influence of other muscles than those passing the joint. Recording of the isometric strength as a pull does not. Utilizing such a test method it is, therefore, possible to exert a greater tension than anticipated from the testing of the torque. It is reasonable to expect that the test method which registers the strength as a pull is more apt to be influenced by a greater degree of skill. In the experiment of HETTINGER and MÜLLER the muscle strength was recorded as a pull, and the skill factor would tend to modify the effect of the training.

The number of subjects used in a training experiment is of importance in evaluating the statistical significance of the results. It was shown in the present experiments as well as by DARCUS and SALTER (1955) that large spontaneous variations take place in muscle strength from time to time. These variations were to a certain extent eliminated in the average of a group. Since the results of HETTINGER and MÜLLER (1953) are founded upon observations of single persons while a group of 10 subjects was used in the present experiments, some question must remain as to the validity of their conclusions.

Conclusions

Only strenuous exercises, not necessarily maximal, are able to increase the isometric strength. However, endurance to isometric work was not influenced by any training program. It was also found that one daily maximum isometric

contraction does not influence the isometric muscle strength. Whether the contractions in the training should be isometric, dynamic or eccentric cannot be definitely stated, but as dynamic work is more comfortable for the subjects, this kind of training may be preferred. The best kind of training program to provide the most favorable increase in strength cannot be given, but the results suggest that an increasing number of contractions will lead to increasing muscle strength.

This work was supported by Miss P. A. Brandt's Foundation. The author is greatly indebted to Mr. P. Rosenfalck, M. Sc. and to Dr. S. Horvath, for their kind help with the statistics in this work and for many suggestive discussions during its progress. The instruments used were constructed in collaboration with Mr. C. Løche Persson.

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Eine Methode zur wiederholten Bestimmung der Gesamthämoglobinmenge und des Blutvolumens von Ratten

Von

BERNHARD TRIBUKAIT

Eingegangen am 3. September 1959

Abstract

TRIBUKAIT, B. *Eine Methode zur wiederholten Bestimmung der Gesamthämoglobinmenge und des Blutvolumens von Ratten.* Acta physiol. scand. 1960. *48.* 417—430. — A modified method for the repeated determination of the total amount of hemoglobin and the blood-volume of rats is described. The animal is kept in a sealed glass container connected to a closed system of circulating air, in which the oxygen-concentration is kept at a constant level by means of absorption of carbon-dioxide and addition of oxygen. The animal remains in the apparatus while a known quantity of CO is added, and a balance is reached between the CO-concentration of the air and the COHb-saturation after about 50 min. From the measured CO-concentration and the volume of the system the CO-quantity absorbed by the animal can be calculated which is dependent on the total amount of hemoglobin and the oxygen-concentration.

The mean error of estimate calculated from duplicate determinations was 4—5 % varying with the size of the animal. The experiment can be repeated on the same animal without any interference with the hematopoiesis. From the total amount of hemoglobin and the hemoglobin-concentration the blood-volume can be calculated. The amount of hemoglobin present in the bone-marrow has been estimated by spectrophotometrical analysis at 0.01 g Hb/g bone-marrow which corresponds to give an average systematic error of less than 3 %. The error by other substances, such as myoglobin, appears not to be of significance.

Blutvolumenbestimmungen bei Kleintieren werden heute in grossem Umfang durchgeführt. Fast alle Methoden gehen von der Verdünnung in die Blutbahn injizierter Farbstoffe (Evans-Blue, Kongorot), radioaktiv gezeichneten Plasma-

eiweisses oder radioaktiv gezeichneter Erythrocyten aus. Da diese Methoden mit der Entnahme einer für kleine Tiere relativ grossen Blutmenge verbunden sind, sind wiederholte Blutvolumenbestimmungen damit ohne Störung der Hämatopoiese nicht oder kaum möglich; zudem ist die sichere intravasale Applikation der Fremdsubstanzen nur am freigelegten Gefäss möglich.

Im Gegensatz zu einer von SCOTT und BARCROFT (1924) auch bei der Ratte verwendeten CO-Methode, bei der die COHb-Sättigung direkt bestimmt wurde und somit ebenfalls Blut entnommen werden musste, vermeidet ein von GEMZELL und SJÖSTRAND (1954) für Kleintiere ausgearbeitetes Verfahren diese Nachteile. In Analogie zu der von SJÖSTRAND (1948) für den Menschen angegebenen Methode wird einem geschlossenen Atemsystem eine bekannte Menge CO zugeführt und die vom Hämoglobin aufgenommene Gesamtmenge CO sowie die Sättigung des Hämoglobins mit CO indirekt durch Analyse der Atemluft bestimmt. Daraus lässt sich die Gesamthämoglobinmenge, das Gesamtblutvolumen mit Hilfe der Hämoglobinkonzentration errechnen.

Die Methode ist, um eine grössere Messgenauigkeit zu erreichen, weiterentwickelt worden, wie nachfolgend beschrieben werden soll.

Allgemeine Voraussetzungen

In einem geschlossenen System mit einer aus Blut bestehenden Flüssigkeitsphase und einer aus CO und O_2 bestehenden Gasphase verteilen sich, nachdem sich ein Gleichgewichtszustand hergestellt hat, CO und O_2 entsprechend der Haldane'schen Gleichung

$$M \times \frac{pCO}{pO_2} = \frac{COHb}{O_2Hb} \cdot \qquad (1)$$

pCO und pO_2 stellen die Partialdrucke von CO und O_2, COHb und O_2Hb die prozentuale Sättigung von Hämoglobin mit CO und O_2 dar. M ist eine Gleichgewichtskonstante.

Ein derartiges Verhalten liegt praktisch in der Lunge beim Atmen von CO und O_2 in einem geschlossenen System vor. Der Partialdruck von CO im Lungenkapillarblut entspricht dem der Alveolarluft (P_{ACO}) und lässt sich aus der Gleichung

$$P_{ACO} = F_{CO} \times (P_B—47) \qquad (2)$$

berechnen. F_{CO} ist der Anteil von CO in der Atmungsluft, P_B der aktuelle Barometerdruck und 47 der Partialdruck der Luftfeuchtigkeit in mm Hg bei 37° C und Wasserdampfsättigung.

In gleicher Weise lässt sich der Partialdruck von O_2 im Lungenkapillarblut approximativ aus der Gleichung des O_2-Partialdruckes der Alveolarluft

$$P_{AO_2} \approx F_{O_2} \times (P_B—47) — 40 \qquad (3)$$

berechnen. F_{O_2} ist der Anteil von O_2 in der Atmungsluft, die Zahl 40 bezeichnet den durchschnittlichen alveolären CO_2-Druck in mm Hg, um den der mittlere O_2-Druck in der Alveole absinkt.

Bei Atmung von Luft und CO liegt praktisch alles Hämoglobin bis auf das CO-Hämoglobin als O_2-Hämoglobin vor. O_2Hb beträgt also 100 % – COHb.

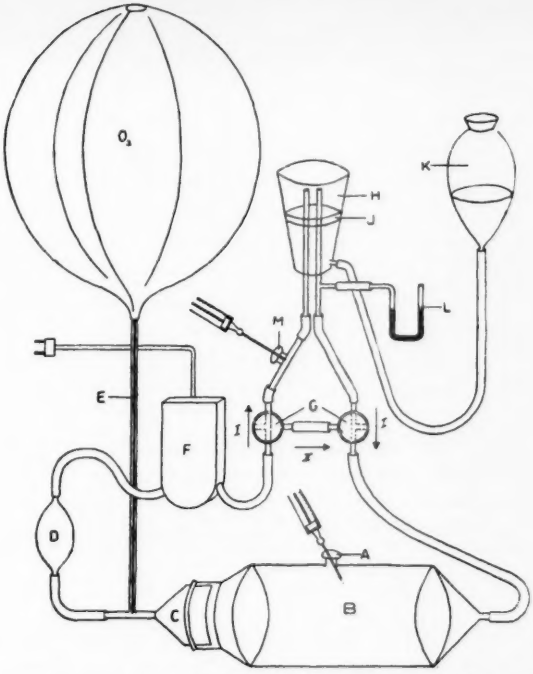

Abb. 1. Halbschematische Abbildung der Apparatur. Beschreibung s. Text.

Führt man die Gleichungen (2) und (3) in die Haldane'sche Gleichung ein, ergibt sich für die Sättigung von Hb mit CO

$$\text{COHb} = \frac{100 \times M \times P_{ACO}}{M \times P_{ACO} + P_{AO_2}}. \qquad (4)$$

Die Gesamthämoglobinmenge (Tot.-Hb in g) lässt sich schliesslich mit Hilfe der Gleichung

$$\text{Tot.-Hb} = \frac{100 \times V_{CO}}{1.34 \times \text{COHb}} \qquad (5)$$

bestimmen. V_{CO} ist die vom Organismus aufgenommene Menge CO, umgerechnet auf 0° und 760 mm Hg; 1.34 ist die Menge CO in ml, die 1 g Hämoglobin zu binden vermag.

Apparatur und Gang der Bestimmung

Innerhalb eines geschlossenen Atemsystems vermag sich nur unter folgenden Voraussetzungen ein Gleichgewicht zwischen O_2 und CO einerseits und O_2Hb

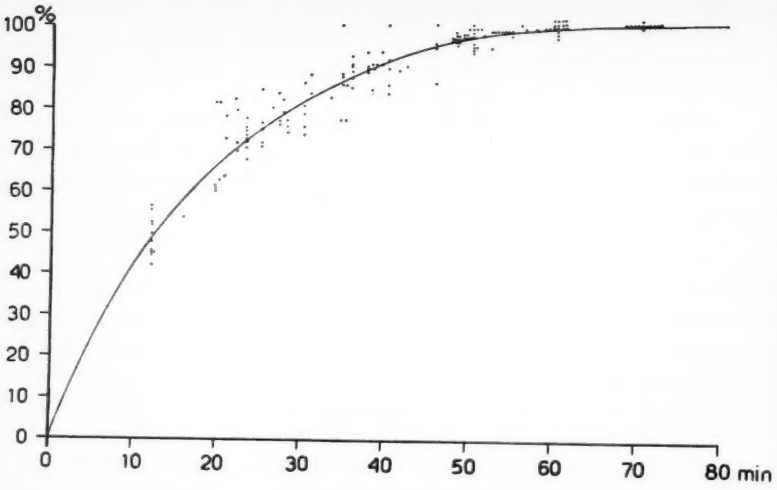

Abb. 2. Aufgenommene Menge CO in % der Gesamtaufnahme (Abszisse) während 80 Minuten (Ordinate). Werte von 20 Tieren verschiedener Grösse.

und COHb andererseits einzustellen: Während der Versuchszeit darf sich die O_2-Konzentration der Atmungsluft nicht verändern; das Volumen des Atemsystems muss konstant bleiben; es darf sich kein CO_2 anreichern.

Die in Abb. 1 halbschematisch wiedergegebene Apparatur erfüllt diese Forderungen unter Anwendung eines zirkulierenden Systems weitgehend. Das Versuchstier befindet sich in der Glaskammer B, die durch den Schliff C verschlossen wird. Die Membran-Pumpe F sorgt für ständige Zirkulation der Luft. Das vom Tier abgegebene CO_2 wird durch KOH in dem Gefäss D absorbiert. Da gleichzeitig O_2 verbraucht wird, entsteht im System ein Unterdruck, der durch Zufuhr von O_2 aus einem Gummibeutel kompensiert wird. Als sicheres und schwereloses Ventil dient die Kapillare E. Während des Versuches sind die Dreiweghähne G in Richtung der Pfeile I gestellt, wodurch das im Nebenschluss liegende Gefäss H durchströmt wird. Nach Abschluss des Versuches werden die Dreiweghähne in Richtung des Pfeiles II gedreht und damit das Gefäss H von der Zirkulation abgeschaltet. Das Tier kann herausgelassen und der Behälter B gereinigt werden, während durch die Gummikappe M mit einer paraffinierten Spritze Gasproben zur Analyse entnommen werden. Druckveränderungen dabei werden durch das Niveaugefäss K unter Kontrolle des Manometers L vermieden. Die Paraffinschicht J verhindert einen Kontakt zwischen dem Wasser des Niveaugefässes und dem Gas. An dem Tierbehälter befindet sich eine weitere Gummikappe A, durch

Tab. I. Konzentrationen von O_2 und CO (ohne Zufuhr von CO) nach 70 Minuten in verschiedenen Versuchsserien

% O_2	% CO × 100,000
20.5 ± 0.12	1.58 ± 0.36
(17.3 — 24.0)	(0.00 — 8.0)
n = 104	n = 50

die, ebenfalls mit einer paraffinierten Spritze, dem System 0.9829 ± 0.00018 ml eines 97%-igen CO zugeführt werden. Das Volumen des gesamten Systems beträgt ca. 1,200 ml. Um eine Diffusion von CO durch die Pumpe zu vermeiden, ist deren Gummimembran durch eine Kunststoffmembran verstärkt worden.

Die CO-Konzentration wird nach der Methode von LINDERHOLM und SJÖSTRAND (1956) (Oxydation von CO → CO_2 über Hopkalit, Messen der freigesetzten Wärmemenge thermoelektrisch), die Sauerstoffkonzentration nach Haldane bestimmt. Wie aus Abb. 2 zu entnehmen ist, hat sich unter den gegebenen Voraussetzungen ein Gleichgewicht nach etwa 50 Minuten eingestellt. Zur Sicherheit bleiben die Tiere insgesamt 70 Minuten in der Apparatur.

Nach dieser Zeit betrug in 104 Versuchen die mittlere O_2-Konzentration 20.5 % ± 0.12, während sich in 50 Versuchen ohne Zufuhr von CO keine nennenswerten Mengen CO oder anderer flüchtiger Stoffe, die sich bei der Analyse wie CO verhalten, gebildet hatten (siehe Tab. I).

Die prozentuale Sättigung des Hämoglobins mit CO und die Gesamthämoglobinmenge werden nach den eingangs entwickelten Gleichungen (4) und (5) berechnet. Die aufgenommene Menge CO (V_{CO}), umgerechnet auf 0° und 760 mm Hg, ergibt sich aus der injizierten Menge CO (V_{CO} inj.), der gefundenen CO-Konzentration (F_{CO}), dem Volumen des Apparates ($V_{App.}$) und dem Tiervolumen abzüglich dem Volumen der Lunge des Tieres (V_T). Das Tiervolumen (— Lungenvolumen) in ml entspricht dem Tiergewicht in g. Es gilt dann

$$V_{CO} = V_{CO}\text{inj.} - (V_{App.} - V_T) \times F_{CO}.$$

Bestimmung des Faktor M

Der Faktor M leitet sich aus der Haldane'schen Gleichung

$$M = \frac{p\,O_2 \times COHb}{p\,CO \times O_2Hb}$$

ab. Er ist von 24 Ratten in vivo bestimmt worden.

Tab. II. Einzeldaten der gefundenen CO- und O_2-Partialdrucke des Systems, der daraus berechneten alveolaren Partialdrucke und der gefundenen COHb-Sättigung. M berechnet aus den alveolaren Partialdrucken und der COHb- bzw. O_2Hb-Sättigung, M' berechnet aus den Partialdrucken des Systems

Tier Nr.	pCO mmHg (System)	pO_2 mmHg (System)	P_{ACO} mmHg	P_{AO_2} mmHg	COHb %	M	M'
1	0.1130	146	0.1059	96	21.2	243.9	347.6
4	0.1819	161	0.1707	111	22.5	188.8	257.1
5	0.1141	147	0.1069	97	19.9	225.4	320.1
10	0.0917	141	0.0860	92	14.0	174.1	250.3
11	0.1548	158	0.1453	109	22.8	221.6	301.4
12	0.1327	161	0.1246	111	20.5	229.7	312.9
13	0.1374	155	0.1289	106	21.4	223.9	307.1
14	0.1008	143	0.0945	95	20.4	257.6	363.6
15	0.1437	159	0.1349	109	22.2	230.6	315.7
16	0.0928	144	0.0882	95	15.0	190.1	273.8
17	0.1274	157	0.1195	107	20.2	226.7	311.9
19	0.1328	164	0.1246	114	20.4	234.5	316.5
21	0.1444	156	0.1356	106	21.3	211.6	292.4
70	0.1219	138	0.1143	89	22.0	219.6	319.3
71	0.1296	157	0.1215	107	20.8	231.3	318.2
74	0.1377	156	0.1292	106	20.7	214.2	296.8
75	0.1164	153	0.1092	104	19.8	235.1	324.5
76	0.1295	132	0.1210	83	22.8	202.6	301.0
77	0.1908	164	0.1780	113	26.8	232.4	314.7
78	0.1293	157	0.1210	109	18.3	201.7	272.6
82	0.1230	155	0.1152	105	19.8	225.0	311.1
84	0.1706	156	0.1599	106	23.7	205.0	284.0
85	0.1290	150	0.1210	101	22.6	243.7	339.5
93	0.1147	143	0.1076	94	20.7	228.0	325.4

Das Versuchstier befand sich wie bei einer gewöhnlichen Gesamthämoglobinbestimmung in der Apparatur. Nach 70 Minuten wurde es herausgelassen, worauf sofort von seiner Schwanzspitze 2 Pipetten mit je 0.1 ml Blut luftfrei gefüllt wurden. Ferner wurde die relative Hämoglobinkonzentration bestimmt. (Methodik siehe unter Bestimmung des Gesamtblutvolumens.) Die Blutprobe wurde bis zur Analyse unter Luft- und Lichtabschluss bei 4° aufbewahrt. Die Sättigung von Hämoglobin mit CO wurde nach der Methode von LINDERHOLM, SJÖSTRAND und SÖDERSTRÖM (1959) gemessen: In einer modifizierten van Slyke-Pipette wurden 0.1 ml Blut mit Saponinlösung hämolysiert und das an Hämoglobin gebundene CO in vacuo durch Zusatz von $KH(JO_3)_2$ freigesetzt. Dieses wurde wiederum nach LINDERHOLM und SJÖSTRAND (1956) bestimmt und au. 760 mm Hg und 0° umgerechnet. Bei bekannter Hämoglobinkonzentration und unter der Annahme, dass 1 g Hämoglobin 1.34 ml CO zu binden vermag,

lässt sich die COHb-Konzentration der Blutprobe errechnen. Aus Tab. II sind von einer Versuchsreihe die im System gefundenen Partialdrucke O_2 und CO, die sich daraus ableitenden alveolaren Partialdrucke CO und O_2 und die Werte für die COHb-Sättigung ersichtlich. Aus den alveolaren Gasdrucken und der COHb- bzw. O_2Hb-Sättigung wurden M-Werte errechnet, die dem Verhalten eines reinen Gas-Blutsystems etwa entsprechen. Ferner sind lediglich aus dem Gasdrucken des Systems und der COHb- bzw. O_2Hb-Sättigung Gleichgewichtskonstanten — mit M' bezeichnet — berechnet worden. Für die Doppelbestimmungen der COHb-Sättigung betrug der Variationskoeffizient $\pm$ 3.8 %. Der aus den alveolaren Gasdrucken berechnete Faktor M hat einen mittleren Wert von 220.8 $\pm$ 3.9.

Die Rolle von Myoglobin und nicht-zirkulierendem Hämoglobin bei der Bestimmung des Blutvolumens

Myoglobin und geweblich fixiertes Hämoglobin vermögen neben dem zirkulierenden Hämoglobin CO zu binden. Die Grössenordnung der von diesen Systemen aufgenommenen CO-Menge soll nachfolgend abzuschätzen versucht werden.

1. Myoglobin

Wie beim Hämoglobin besteht zwischen der prozentualen Sättigung von Myoglobin mit CO und O_2 und deren Partialdrucken die Beziehung

$$\frac{\text{CO Mgb.}}{\text{O}_2\ \text{Mgb.}} = \text{K} \times \frac{\text{p CO}}{\text{p O}_2},$$

oder für die Sättigung CO Mgb:

$$\text{CO Mgb} = \frac{100 \times \text{K} \times \text{p CO}}{\text{K} \times \text{p CO} + \text{p O}_2}.$$

Es sei eine 275 g schwere Ratte betrachtet, die bei einem alveolaren pCO von 0.154 mm Hg und einem alveolaren pO_2 von 113 mm Hg 0.680 ml CO aufgenommen hat. Für den CO-Partialdruck in der Muskelzelle wird nach CAMPBELL (1929) 1/3 des alveolaren pCO, also 0.0513 mm Hg angenommen, für den intrazellulären O_2-Partialdruck 10 mm Hg. K sei 13.8 (THEORELL 1934, Pferdemyoglobin). Die Sättigung COMgb beträgt dann 6.6 %.

Bei einer Gesamtmyoglobinmenge von 0.1 g (DRABKIN 1948) und einer O_2-bezw. CO-Kapazität von 1.3 ml/g Myoglobin sind 0.008 ml CO vom Myoglobin aufgenommen worden, d. h. etwa 1 % der gesamten aufgenommenen CO-Menge.

Nach einer Entnahme von etwa 1/3 der Blutmenge des Tieres ist bei einem alveolaren pO_2 von 106 mm Hg der alveolare pCO auf 0.206 mm, der intramuskuläre pCO auf 0.0687 mm Hg gestiegen und die gesamte aufgenommene CO-Menge auf 0.627 ml gesunken. Bei gleichem intrazellulären O_2-Partial-

druck von 10 mm Hg ist jetzt die Sättigung COMgb 8.7 %, die vom Myoglobin aufgenommene CO-Menge beträgt 0.01 ml, d. h. rund 2 % der gesamten aufgenommenen CO-Menge.

2. Nicht-zirkulierendes Hämoglobin

Träger des geweblich fixierten Hämoglobins ist das Knochenmark. Die Absolutmenge von Hämoglobin im Knochenmark ergibt sich aus dem Gesamtvolumen des Knochenmarks und seiner Hämoglobinkonzentration. Für das Gesamtvolumen werden 2 % des Körpergewichtes angenommen (HUDSON 1958; Meerschweinchen. Von FAIRMAN und CORNER 1934 für die Ratte angegebene Werte von 3 % erscheinen weniger verlässlich). Die Hämoglobinkonzentration ist vom Knochenmark des Femur der Ratte bestimmt worden. Um zirkulierendes Hämoglobin möglichst aus dem Knochenmark zu entfernen, wurde den Tieren in Narkose bis zum Herzstillstand Blut entnommen und gleichzeitig Kochsalzlösung infundiert. Versuche mit Cr^{51}-gezeichneten Erythrocyten ergaben, dass dadurch rund 75 % des im Knochenmark vorhandenen zirkulierenden Hämoglobins herausgespült werden. Das danach im Knochenmark verbleibende Hämoglobin wurde in 0,1 %-iger Na_2CO_3-Lösung extrahiert und als Pyridin-Hämochromogen spektrophotometrisch bei einer Wellenlänge von $\lambda = 557$ und $\lambda = 540$ mμ (Beckman DU) quantitativ bestimmt (PAUL, THEORELL und ÅKESON 1953).[1] Die Hämoglobinmenge von 8 verschiedenen Knochenmarksproben (abzüglich der zirkulierenden Hämoglobinmenge) lag zwischen 0.00891 und 0.02621 g Hb/g Knochenmark (Mittelwert 0.01495 ± 0.002). Ausgehend von diesem Mittelwert hat die 275 g schwere Ratte mit einem Gesamtknochenmark von 5.5 g rund 0.082 g Hämoglobin dort fixiert. Die O_2-Sättigung des Knochenmarkes entspricht etwa der des venösen Blutes (GRANT und ROOT 1947, GRANT 1948, BERK *et al.* 1948, SCHWARTZ und STATS 1949). Unter Berücksichtigung des vom menschlichen Blut abweichenden Verlaufes der O_2-Dissoziationskurve des Rattenblutes (JONES, MAEGRAITH und SCULTORPE 1950) wird ein O_2-Partialdruck von 70 mm Hg und ein CO-Partialdruck von 0.095 mm Hg (alveolarer $pCO = 0.154$ mm Hg) angenommen. Die COHb-Sättigung des Knochenmarkhämoglobins beträgt dann 23.1 %. Insgesamt hat das Knochenmark 0.0254 ml CO aufgenommen, d. h. 3.7 % der vom Gesamtorganismus gebundenen CO-Menge.

Methodenfehler

Die Grösse des Methodenfehlers (Summe aller experimentellen Fehler) ist an Hand von Doppelbestimmungen ermittelt worden. In einigen routinemässig durchgeführten Versuchsserien sind alle, innerhalb von 48 Stunden gewonnenen Werte der einzelnen Tiere dazu herangezogen worden. Bei 50 Doppelbestimmungen von Tieren über 250 g Körpergewicht betrug der Variationskoeffizient

[1] Für die Unterstützung bei der Bestimmung der Werte danke ich Herrn Dr. K. G. Paul, Medizinisches Nobelinstitut, Biochemische Abteilung, Stockholm.

Tab. III. Totalhämoglobin von Ratten verschiedener Versuchsserien, bestimmt vor und nach der Entnahme einer bekannten Hämoglobinmenge

Anzahl	Total-Hb.		Differenz 1. — 2.	Abgenommene Hb-Menge
	1. vor Blutentnahme	2. nach Blutentnahme		
n = 10	2.55 ± 0.11	1.88 ± 0.09	0.67	0.680 ± 0.006
n = 9	2.67 ± 0.07	2.00 ± 0.07	0.67	0.694 ± 0.010
n = 10	2.69 ± 0.08	1.98 ± 0.09	0.71	0.678 ± 0.009

3.5 % entsprechend 0.0801 g Hämoglobin bei einer durchschnittlichen Hämoglobinmenge von 2.29 g. 50 Doppelbestimmungen von Tieren unter 250 g ergaben einen Variationskoeffizienten von 4.2 % oder 0.0542 g Hämoglobin bei einer durchschnittlichen Hämoglobinmenge von 1.29 g. In die Grösse dieses Methodenfehlers gehen natürliche Veränderungen der Hämoglobinmenge ein. Beim wachsenden Tier pflegt der zweite Wert der Doppelbestimmung etwas höher als der erste zu sein; der Unterschied ist jedoch statistisch nicht zu sichern. Bei alten Tieren können gelegentlich spontane Schwankungen auftreten, die bei wiederholten Bestimmungen deutlich werden. In einer Serie ausgesuchter Tiere und bei sehr sorgfältiger Technik lag der Methodenfehler bei 2 %.

Anwendungsbeispiel

Im Hinblick auf die Methode interessiert es, die Hämoglobinmenge vor und nach einer Blutentnahme zu bestimmen. Entsprechen die nach der Entblutung gemessene und die entnommene Hämoglobinmenge der vor der Blutentnahme bestimmten, ist es recht wahrscheinlich, dass diese einen absoluten Wert darstellt.

Bei Tieren dreier Versuchsgruppen wurde deshalb zu verschiedener Zeit unter peinlicher Vermeidung von Blutungen die V. cava inf. freigelegt und etwa 1/3 der Gesamtblutmenge entnommen. Aus Tab. III sind die Mittel der vor und nach der Blutentnahme bestimmten Gesamt-Hb-Werte, ihre Differenzen sowie die mittleren abgenommenen Hb-Werte ersichtlich. Die Differenzen stimmen mit der abgenommenen Menge gut überein.

Bestimmung des Gesamtblutvolumens, des Gesamtplasma- und Gesamterythrocytenvolumens

Das Gesamtblutvolumen (Tot.Bl.Vol. in ml) leitet sich aus der Gleichung

$$\text{Tot. Bl.Vol.} = \frac{\text{Hb} \times 100}{\text{rel.Hb}} \text{ ab.}$$

Tab. IV. Hämatocrit und Hämoglobinkonzentration des peripheren, arteriellen und venösen Blutes, Mittelwerte von 11 Ratten

Hämatocrit %			Hämoglobin g%		
1. peripher	2. arteriell	3. venös	1. peripher	2. arteriell	3. venös
45.2 ± 1.0	39.9 ± 1.4	39.8 ± 0.9	14.2 ± 0.3	12.3 ± 0.2	12.1 ± 0.3
p 1—2 0.05—0.02			p 1—2 0.001		
p 1—3 0.01—0.001			p 1—3 0.001		
p 2—3 0.9			p 2—3 0.5—0.4		

Für das Gesamtplasmavolumen (Tot.Pl.Vol. in ml) gilt

$$\text{Tot. Pl.Vol.} = \frac{\text{Tot.Hb} \times 100}{\text{rel.Hb}} \times \frac{100 - \text{Hct.}}{100},$$

für das Gesamterythrocytenvolumen (Tot.Ery.Vol. in ml)

$$\text{Tot. Ery.Vol.} = \frac{\text{Tot.Hb} \times 100}{\text{rel.Hb}} \times \frac{\text{Hct.}}{100}.$$

Die relative Hämoglobinkonzentration (rel.Hb) ist in g/100 angegeben. 0.025 ml Blut werden in 5 ml 0.04 %-iger Amoniaklösung hämolysiert, die Konzentration der Lösung wird in einem Beckman-B-Spektrophotometer bei der Wellenlänge $\lambda = 545$ mμ gemessen. Die Methode ist mit Hilfe der van Slyke-Technik unter der Annahme, dass 1 g Hämoglobin 1.34 ml O_2 bindet, standardisiert (SUNDERMAN *et al.* 1953). Der Methodenfehler, der den Fehler beim Pipettieren einschliesst, von 30 Doppelbestimmungen ermittelt, beträgt für eine Einzelbestimmung 2.5 %.

Der Hämatocrit (Hct.) gibt den Erythrocytenanteil einer Blutprobe in % an. Bei der verwendeten Mikromethode (International Hämacrit-Centrifuge, 11,000 Umdrehungen/Minute, 5 Minuten lang) befinden sich bei einem Hämatocrit von über 40 % in dem Erythrocytenanteil noch 2 % Plasma, was bei der Berechnung zu berücksichtigen ist (ESTBORN 1959). Der von 30 Doppelproben bestimmte Methodenfehler beträgt 1.5 %.

Es ist bekannt, dass die Hämoglobinkonzentration und der Hämatocrit der verschiedenen Gefässabschnitte Unterschiede aufweist (FRIEDMAN 1959, WANG 1959). Bei der Ratte ist der Schwanz bevorzugter Körperteil, von dem kleine Blutmengen gewonnen werden. Um eine Auffassung darüber zu erhalten, ob Erythrocyten- und Hämoglobinkonzentration des Schwanzblutes mit der anderer Gefässabschnitte übereinstimmt, sind gleichzeitig vom Blut der Schwanzspitze, der Aorta abdom. und der V. cava inf. die Hämoglobinkon-

zentration und der Hämatocrit bestimmt worden. Wie aus Tab. IV ersichtlich ist, unterscheiden sich Hämatocrit und Hämoglobinkonzentration des Schwanzblutes signifikant von den Werten des arteriellen und venösen Blutes. Zwischen arteriellem und venösem Blut besteht kein statistisch gesicherter Unterschied. In 50 derartigen Versuchen war der periphere Hämatocrit (Schwanz) gegenüber dem zentralen Hämatocrit um durchschnittlich 14.5 ± 0.96 %, die periphere Hämoglobinkonzentration um durchschnittlich 12.4 ± 1.14 % höher. Dieser Unterschied besteht auch bei anämischen und polycythämischen Ratten.

Diskussion

Absicht der dargelegten Methode ist, wiederholt die Gesamthämoglobin- oder Blutmenge der Ratte zu bestimmen, ohne durch Injektion von Fremdsubstanzen oder Blutentnahme deren Erythropoiese zu stören. Da zum Errechnen des Blutvolumens die Hämoglobinkonzentration notwendig ist, ist dieses Ziel im wörtlichen Sinn nur hinsichtlich der Hämoglobinmenge erreichbar. Die zur Bestimmung der Hämoglobinkonzentration notwendige Blutmenge von maximal 0.025 ml Blut ist jedoch gegenüber dem Gesamtblutvolumen so gering, dass eine Störung der Erythropoiese dadurch ausgeschlossen werden kann.

Grundsätzlich alle Methoden zur indirekten Blutvolumenbestimmung gehen von der Verdünnung einer bekannten Menge einer Fremdsubstanz aus, die dem Blute zugeführt wird. Das gilt auch für die CO-Methode, wobei die Applikation von CO über ein geschlossenes Atemsystem erfolgt. Das zugeführte CO wird nun aber nicht quantitativ vom Blut aufgenommen, sondern es stellt sich ein Gleichgewicht zwischen den Partialdrucken CO und O_2 der Atemluft und der COHb- bezw. O_2Hb-Konzentration ein. Es muss deshalb neben dem Grad der Verdünnung im Blut die aufgenommene CO-Menge aus der CO-Konzentration und der Grösse des Atemsystems bestimmt werden. Dieser gegenüber den Injektionsmethoden zweifellose Nachteil lässt sich aber dahingehend vorteilhaft ausnutzen, dass man gleichzeitig indirekt die COHb-Sättigung des Blutes aus den Gasdrucken CO und O_2 der Atemluft und einer Gleichgewichtskonstanten bestimmt. Damit wird die direkte Blutgasanalyse umgangen.

Dem idealen Verhalten eines reinen Gas-Blutsystems kommt man am nächsten, wenn man von den Gasdrucken der Alveolarluft ausgeht. Der zur Bestimmung des alveolaren O_2-Druckes wichtige mittlere alveolare CO_2-Druck wurde mit 40 mm Hg angenommen. Bei der narkotisierten Ratte haben Blood, Elliott und d'Amour (1946) einen CO_2-Druck von 40.2 mm Hg gefunden, während Rahn und Canfield (1955) bei der nicht narkotisierten Ratte aus der Gaszusammensetzung subcutan injizierten Stickstoffs einen alveolaren CO_2-Druck von 36 mm Hg errechnet haben. Wegen der Unsicherheit dieses Wertes ist darauf verzichtet worden, eine Korrektur für den respiratorischen Quotien-

ten, der bei der Ratte etwa 0.8 beträgt (GRIFFITH und FARRIS 1942), vorzunehmen.

Die in vivo bestimmte Gleichgewichtskonstante mag deshalb mit dem in vitro bestimmten Absolutwert nur annäherungsweise übereinstimmen. An den mit diesem empirischen Faktor und den angenommenen Alveolardrucken berechneten COHb-Werten ändert sich indessen dadurch nichts, da die berechneten Gasdruckwerte der Alveolarluft in einer konstanten Beziehung zum Gasdruck des Lungenkapillarblutes stehen. Ebensogut ist es auch möglich, lediglich aus den Partialdrucken der Systemluft und einer für diesen Fall bestimmten Gleichgewichtskonstanten (M'), die in unseren Versuchen 307.4 ± 5.5 beträgt, die COHb-Sättigung zu bestimmen; denn genauso wie die Gasdrucke der Alveolarluft stehen auch die Gasdrucke der Systemluft zum Lungenkapillarblut in einem konstanten Verhältnis.

Im Gegensatz zur Methode beim Menschen ist Luftatmung bevorzugt worden. Einmal ist es unnötig, aus messtechnischen Gründen die CO-Konzentration zu erhöhen, die bei O_2-Atmung etwa 5 mal grösser sein muss, um dieselbe COHb-Sättigung wie bei Luftatmung zu erhalten. Die Empfindlichkeit der Messapparatur wird bei den verwendeten CO-Mengen von 0.01 bis 0.0025 ml bei weitem nicht ausgeschöpft. Es ist ausserdem einfacher, mit Luft zu arbeiten. Vor allen Dingen ist die Empfindlichkeit der Methode, kleine Schwankungen der Hämoglobinmenge zu erfassen, bei Luftatmung grösser als bei O_2-Atmung: Angenommen, die Gesamthämoglobinmenge steigt und die CO-Konzentration der Luft sinkt um 10 %, so verursacht derselbe Hämoglobinanstieg bei O_2-Atmung nur einen 2 %-igen Abfall der CO-Konzentration.

Während der Versuchszeit sinkt die O_2-Konzentration innerhalb der Apparatur etwas ab. Dafür ist im wesentlichen die Wärmeentwicklung des Tieres verantwortlich, wodurch sich das Gas im System etwas ausdehnt und relativ weniger O_2 einströmt. Bei sehr grossen Tieren und niedriger Zimmertemperatur kann es daher notwendig werden, vor Versuchsbeginn den Tierbehälter etwas aufzuwärmen, etwa durch einen Gummibeutel mit warmem Wasser.

Das im Knochenmark fixierte Hämoglobin vermag rund 4 % der gesamten aufgenommenen CO-Menge zu binden. Dieser theoretisch errechnete Wert kann in praxi jedoch nicht erreicht werden. Ebenso wie die Sättigung von Hb mit CO in der Lunge ist die Umlagerung des an zirkulierendes Hämoglobin gebundenen CO an das im Knochenmark fixierte Hämoglobin unter anderem abhängig von der Grösse und Durchblutung der austauschenden Fläche, der Kinetik der Dissoziation von COHb und dem Druckgefälle von CO zusammen mit dem Druckverhalten von O_2 und CO_2. Die quantitative Bedeutung der einzelnen Grössen lässt sich schwer abschätzen. Lediglich vom Druckgefälle ist bekannt, dass es sein Maximum nicht vor 50 Minuten erreicht hat. Wahrscheinlich macht die CO-Aufnahme im Knochenmark bei der Versuchszeit von 70 Minuten nur einen kleinen Teil der Gesamtmenge CO aus, die theoretisch aufgenommen werden kann.

Eine allgemeine Vorstellung können Versuche über die fötale CO-Aufnahme bei 30 minutiger CO-Atmung der Mutter vermitteln. Trotz der im Verhältnis zum Knochenmark sicherlich besseren Gasaustauschbedingungen durch die Placenta nimmt der Fötus nur etwa 10 % der CO-Menge auf, die er maximal aufnehmen könnte (GEMZELL, ROBBE und STRÖM 1958).

Diese Überlegungen gelten auch für Myoglobin, das indessen rein quantitativ auch bei der theoretisch berechneten maximal möglichen CO-Aufnahme ohne praktische Bedeutung für die Methode ist.

Die Resultate der Ausblutungsversuche scheinen dafür zu sprechen, dass die mit der Methode gewonnenen Werte Absolutwerten recht nahe kommen. Über die Ergebnisse einer vergleichenden Untersuchung der Hämoglobinmenge mit CO und radioaktiv gezeichneten Erythrocyten soll an anderer Stelle berichtet werden (ENGSTEDT, PERIĆ und TRIBUKAIT 1959).

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Acta physiol. scand. 1960. 48. 431—438

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Quantitative Studies on the Anaphylactic Mast-Cell Reaction In Vivo in the Guinea Pig

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Abstract

BORÉUS, L. O. *Quantitative studies on the anaphylactic mast-cell reaction in vivo in the guinea pig.* Acta physiol. scand. 1960. *48.* 431—438. — The anaphylactic reaction of tissue mast cells was studied quantitatively *in vivo* in the nasal mucosa of anaesthetized guinea pigs. Rising doses of antigen, given intraarterially or intravenously, caused proportional degrees of mast-cell depletion in the tissue, as well as increasing intensity of the anaphylactic shock symptoms. Topical administration of different concentrations of antigen on the mucosa produced proportional degrees of mast-cell disappearance, but gave no shock symptoms. A total mast-cell loss in the mucosa was never seen, even following very high doses of antigen. The cell reaction was temporally closely connected to the onset of the anaphylactic shock symptoms. Maximum was found in 1 minute after intraarterial, and in 2 minutes after topical administration of antigen. The cell reaction and the corresponding anaphylactic shock could not be produced until the 9th day following the first sensitizing dose, but both could be provoked during more than 11 months after sensitization. The conclusion is drawn that the anaphylactic reaction in a guinea-pig tissue is quantitatively reflected in the disappearance of the tissue mast cells.

Quantitative experiments *in vitro* on isolated guinea-pig tissues have shown that the anaphylactic appearance of both histamine and 'slow reacting substance' is correlated to the initial mast-cell count and to the anaphylactic mast-cell disappearance (BORÉUS and CHAKRAVARTY 1960). The degree of mast-cell destruction is thus proportional to the intensity of the anaphylactic reaction *in vitro*. This prompted the present investigation, where the anaphylactic mast-cell reaction has been studied on a quantitative basis *in vivo*, using the nasal mucosa of anaesthetized guinea pigs.

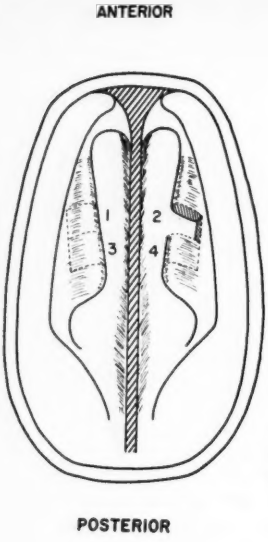

Fig. 1. Schematic drawing of the lower part of the nasal cavity in the guinea pig. The wing-shaped process of the inferior nasal concha is seen on either side of the nasal septum. The dotted lines indicate four specimens, one of which (No. 2) has been removed.

Methods

Guinea pigs, male or female, weighing 250—300 g, were anaesthetized with pentobarbital (about 40 mg/kg intraperitoneally). After insertion of a tracheal cannula, the skin of the dorsum of the nose was removed and two incisions made in the bone on each side of the nasal septum. The bone covering the nasal cavity was removed, and, after hemostasis, the details of the cavity could be studied in the dissecting microscope. The long inferior nasal concha, which originates anteriorly near the nostril, could be seen on each side of the intact septum (Fig. 1). Control specimens were taken from the middle part of the concha on one side. After administration of the drug to be tested, either parenterally or topically, the general symptoms of the animal were noted, and, after a given time, test specimens from the corresponding part of the contralateral concha were taken. Usually two control specimens and two test specimens were taken. The pieces had a size of 2—3 mm in diameter.

The tissue was fixed in a 4 per cent solution of lead subacetate in 50 per cent v/v ethanol with 1 per cent v/v acetic acid. Frozen sections, 30 μ thick, were stained in 0.5 per cent w/v toluidine blue solution in water. The best metachromasia was found when this solution was acidified (with HCl) to pH = 4.

The area of each section was estimated by means of an ocular micrometer; the mean area of the sections was about 7 mm². The total number of mast cells in two or three sections for each specimen was counted at a magnification of 360× and expressed as the number of cells per square unit for each specimen (1 square unit = 0.256 mm²). Two such values were compared, one for the control specimen and one for the corresponding test specimen from the other side, and the difference was expressed as a percentage decrease from the control. This value is called "the mast-cell disappearance" and represents the sensitivity of the cells to the administered drug.

Sensitization of the guinea pigs was produced by a subcutaneous injection of 100 mg egg albumin in 1 ml saline, followed 3 days later by the same dose, given intra-

Table I. Mast-cell frequency in the nasal mucosa of the guinea pig

	Sensitized 63 specimens in 36 animals	Non-sensitized 37 specimens in 20 animals	Total 100 specimens in 56 animals
Number/0.256 mm²	2.41 ± 0.17	2.42 ± 0.29	2.41 ± 0.15
Stand. deviation	± 1.39	± 1.77	± 1.53

peritoneally. Intraarterial administration was made in the common carotid artery. Intravenous injection was made in the jugular vein. All injection volumes were 1 ml and the injection time 1 minute (in the time-effect experiments 30 sec). In topical administration, the test solution was applied directly to the mucous membrane by means of a small pipette.

Results

Anaphylactic changes of the mast cells. The tissue mast cells in sensitized guinea pigs reacted to antigen administration with loss of the metachromatic stainability, so that they ultimately disappeared from the tissue. Some mast cells showed varying degree of fragmentation. This is the same cell reactions as observed in anaphylaxis *in vitro* (BORÉUS and CHAKRAVARTY 1960). Since not all mast cells seem to undergo fragmentation prior to disappearance (BORÉUS, to be published), the most reliable means for quantitative studies in the guinea pig was estimation of the cell population in the different sections. It should be pointed out that the term "disappearance" here is used to signify loss of metachromatic material from the cell with resulting loss of cytoplasmic characteristics. Whether it involves irreversible damage to the cell is not yet known.

Histological characteristics of the inferior concha. The nasal mucous membrane of the inferior concha is thick and rich in blood vessels as well as mast cells. These cells are distributed in the whole tissue with a slightly higher frequency in the submucosal layer. Only the middle part of the concha was used, since the mast-cell frequency was lower in the posterior part and less uniformly distributed in the most anterior part.

The mast-cell frequency in the normal nasal mucosa is seen in Table I. It is shown that there is no difference in mast-cell count between sensitized and non-sensitized animals.

Error of the method. The mean of 50 disappearance values in 25 control animals without treatment was 0.84 % ± 2.41 %. Thus, the mast-cell population was about the same on both sides in the corresponding parts of the nasal mucosa.

General anaphylactic symptoms in the anaesthetized guinea pig. When a sufficient dose of antigen is given intraarterially or intravenously to the anaesthetized, sen-

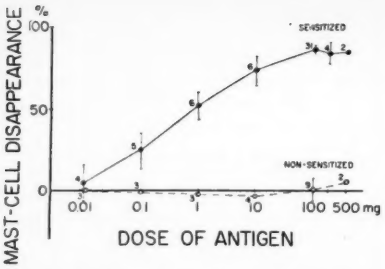

Fig. 2. Effect of different doses of intraarterially administered antigen (egg albumin in saline) on the mast-cell disappearance from the nasal mucosa of the sensitized and non-sensitized guinea pig. The figure at each symbol denotes the number of disappearance values, from which the mean and standard error have been calculated. Injection volume 1 ml, injection time 1 minute. Test specimens taken 5 minutes after start of antigen injection.

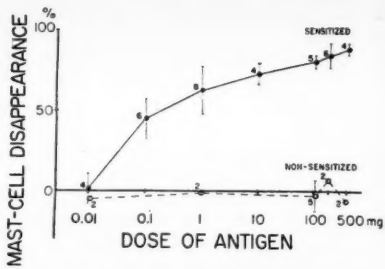

Fig. 3. Effect of different doses of intravenously administered antigen (egg albumin in saline) on the mast-cell disappearance from the nasal mucosa of the sensitized and non-sensitized guinea pig. The figure at each symbol denotes the number of disappearance values, from which the mean and standard error have been calculated. Injection volume 1 ml, injection time 1 minute. Test specimens taken 5 minutes after start of antigen injection.

sitized guinea pig, anaphylactic symptoms occur, usually starting within 20 seconds of the beginning of the injection. Bronchiolar constriction is reflected in distressed respiration, followed by vigorous inspiratory movements, often with open mouth. The high bronchiolar resistance often makes artificial respiration via the tracheal cannula impossible. Cyanosis is evident and the hair of the neck is often raised. After a few minutes the animal defaecates and urinates and stops breathing. Death occurs usually after 4—6 minutes.

Effect of antigen administration. Fig. 2—4 show the mast-cell disappearance for different doses of antigen, given intraarterially, intravenously and topically in sensitized and non-sensitized guinea pigs.

It is seen from Fig. 2 that intraarterial administration of increasing doses of antigen to sensitized animals causes increasing degrees of mast-cell disappearance. Maximal effect, 85.6 % (mean of 31 disappearance values), was reached with the 100 mg dose. Higher doses gave no further increase. A total disappearance of the mast cells was never found, the highest individual value being 97 %. The non-sensitized animals showed no mast-cell reaction upon egg albumin injection. Thus, the disappearance value for 100 mg egg albumin was 0.6 % $\pm$ 7.4 %, which is about the same as the above-mentioned value for the non-treated controls. If this 100 mg value is compared statistically with the different disappearance values for sensitized animals, there is a significant effect for the doses from 1 mg and more ($P < 0.001$). The severity of the anaphylactic shock ran parallel to the mast-cell reaction, the 100—500 mg doses invariably, and the 10 mg dose in most cases giving fatal shock. The 1 mg and 0.1 mg doses gave respiratory symptoms. The 1 mg dose caused death in a few cases but no deaths occurred after the 0.1 mg injections. 0.01

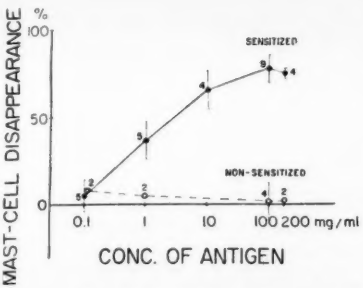

Fig. 4. Effect of different concentrations of topically administered antigen (egg albumin in saline) on the mast-cell disappearance from the nasal mucosa of the sensitized and non-sensitized guinea pig. The figure at each symbol denotes the number of disappearance values, from which the mean and standard error have been calculated. Test specimens taken 10 minutes after antigen administration.

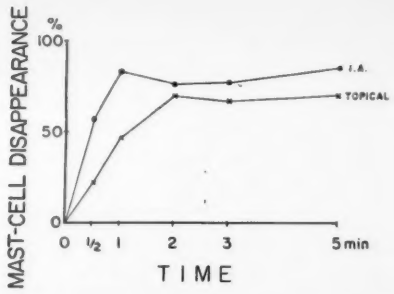

Fig. 5. Time curves for disappearance of mast cells from the nasal mucosa of sensitized guinea pigs following intraarterial injection (100 mg) and topical application (100 mg/ml) of antigen (egg albumin in saline). Injection volume 1 ml, injection time 30 seconds. Mean values of 5 to 7 disappearance values.

mg antigen gave no shock symptoms. None of the non-sensitized animals showed any symptoms after the egg albumin injections.

The dose-response relationship in intravenous injection of antigen is shown in Fig. 3. Maximal effect, 87.5 % disappearance, was recorded in the 500 mg dose. Higher doses were not given. The mast cells of the non-sensitized guinea pig did not reveal any sensitivity to egg albumin. Thus, the 100 mg disappearance value was $-2.4\ \% \pm 9.5\ \%$. If this value is compared with the disappearance values for different doses of antigen to sensitized guinea pigs, the following results are obtained: 500—10 mg: $P < 0.001$; 1 mg: $0.001 < P < 0.01$; 0.1 mg: $0.01 < P < 0.05$; 0.01 mg: $P > 0.5$. This means that intravenous injection is about as effective as intraarterial injection in causing mast-cell disappearance from the nasal mucosa. On the other hand, intravenous injection was slightly more effective in causing anaphylactic death; all animals died from doses of 1 mg and more. Even in the 0.1 mg dose one animal had a fatal shock. The 0.01 mg dose gave no symptoms. Non-sensitized guinea pigs did not show any anaphylactic symptoms when injected with egg albumin.

Topical application of antigen solution to the nasal mucosa gave a dose-dependent degree of mast-cell disappearance in sensitized animals, as shown in Fig. 4. Maximal value was 77.4 %, which was reached with an antigen concentration of 100 mg/ml. Higher concentrations did not augment the effect. No disappearance was found in non-sensitized animals, the 100 mg/ml value being $1.8\ \% \pm 10.7\ \%$. Comparison of this value with the different values found in sensitized animals gave the following P-values: 200—100 mg/ml: $P < 0.001$; 10 mg/ml: $0.001 < P < 0.01$; 1 mg/ml: $0.2 < P < 0.5$.

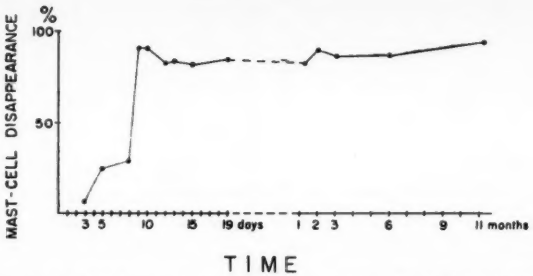

Fig. 6. Time course for development of mast-cell sensitivity. The first sensitizing dose given at day 0, second dose given at day 3. Test dose of antigen (egg albumin in saline) 100 mg, given intraarterially. Injection volume 1 ml, injection time 1 minute. Mean values of 4 to 7 disappearance values.

No anaphylactic symptoms were observed after topical application of antigen to the nasal mucosa of the sensitized or non-sensitized animals. For testing the general sensitivity of the animals, 100 mg antigen was therefore injected intraarterially after the specimens from the topical experiment had been taken. All sensitized animals then evinced fatal anaphylactic shock.

Time-effect experiments. By means of vital fixation through the common carotid artery at different intervals after the beginning of the antigen administration, the mast-cell reaction could be almost instantly interrupted. In this way, time-effect curves for intraarterial and topical administration were established and may be seen in Fig. 5. The onset of the reaction was found to be rapid, and maximal values are reached after 1 minute from the beginning of the intraarterial injection and after 2 minutes when the antigen was applied topically to the mucosa. In the intraarterial experiments, the anaphylactic symptoms started within the first 30 seconds (when more than 50 % of the mast cells had disappeared) and gradually increased. Cardiac arrest as a rule occurred after about 5 minutes.

Time course for development of mast-cell sensitivity. The reactivity of the mast cells in the nasal mucosa to antigen was tested at different intervals after the first sensitizing injection. The test dose of antigen was 100 mg, given intraarterially. The results are presented in Fig. 6. It is seen that full mast-cell reactivity is displayed on the 9th day after the first sensitizing injection, and that it is still present after more than 11 months. Anaphylactic shock also occurred in all experiments from the 9th day. No significant change in mast-cell frequency was observed during the experimental period.

Discussion

The mast-cell reactions *in vivo* in anaphylaxis have so far not been quantitatively investigated, except for the observation of MOTA and VUGMAN (1956) that lungs from guinea pigs which had died in anaphylactic shock

had a significantly lower mast-cell frequency than control animals. The lack of further quantitative investigations in this field is probably due to methodological difficulties encountered in the individual variations of mast-cell frequency and the irregular distribution of the cells in the tissues. Moreover, in the case of the guinea pig, which is the classical species in studying anaphylaxis, the morphological changes of the mast cells in histamine release are not so characteristic and dramatic as, for instance, the mast-cell "disruption" in the rat and hamster.

The symmetrical distribution and the rather high frequency of mast cells in the guinea-pig nasal mucosa makes this tissue well suited for quantitative studies of these cells. The method avoids the erroneous influence of the individual variation of mast-cell population. The inferior concha is well vascularized, which gives a rapid and uniform distribution in the tissue of the administered drugs, both by parenteral and topical routes. Further, by means of the dissecting microscope, the mucosa may be watched *in situ* and with its blood supply intact during the different experimental procedures.

It is seen from the dose-effect curves that a sensitized tissue may show different degrees of anaphylactic mast-cell response *in vivo*. This may be due to variation in sensitivity to antigen in the individual mast cells. This assumption is supported by the finding that a small part of the mast cells did not react, even with very high doses of antigen.

Fatal anaphylactic shock was found after injection of antigen doses of 1—10 mg, which caused a mast-cell disappearance in the nasal mucosa of about 50 %. On the other hand, the topical experiments showed that 77 % of the mast cells may disappear without any general anaphylactic symptoms at all. It is thus evident that one tissue may show a local anaphylactic reaction without any noticeable reaction in other tissues, such as the lung, which, in the guinea pig, is very sensitive to substances liberated in anaphylaxis. Evidently, the shock-producing injections of antigen must have caused mast-cell reaction with histamine liberation in other tissues as well. Since the anaphylactic symptoms after parenteral administration of antigen were parallel to the mast-cell reaction in the nasal mucosa, it may be assumed that a proportional cell reaction was produced in the lung. From the figures of MOTA and VUGMAN (1956) it may be calculated that the intracardial injection of 100 mg antigen to sensitized guinea pigs, which always gave fatal shock, decreased the mast-cell count in the lung to about 1/3 of the controls.

At what stage of the disintegration process the mast cell gives off histamine (and other active principles) is at present not known. The loss of metachromatic material (*i. e.* the disappearance of the cell) is temporally closely connected to the anaphylactic symptoms. Thus, in vital fixation following intra-arterial antigen administration, more than 50 % of the cells had disappeared from the nasal mucosa in 30 seconds. Since the anaphylactic shock symptoms

started within about the same period, it seems that the disappearance reaction occurs in close temporal connection to the histamine liberation.

It is well known that the guinea pig needs a little more than one week to develop active anaphylactic sensitivity. In this paper it is shown that the mast-cell reaction could not be elicited until the 9th day following the first sensitizing injection and that the time of latency in development of general anaphylactic sensitivity and of mast-cell sensitivity is the same. In their experiments on rat mast cells *in vitro*, HÖGBERG and UVNÄS (1958) found that a marked sensitivity of the cells to antigen developed within 10 days after sensitization of the animals.

All results in this paper are consistent with the idea of the mast-cell disintegration as an essential stage in the anaphylactic reaction. Since the disappearance of tissue mast cells bears strong quantitative and temporal correlation to antigen administration and to the anaphylactic shock symptoms, it may be concluded that this cell reaction is a convenient means for quantitative studies of the anaphylactic sensitivity of the guinea pig under various conditions *in vivo*.

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Acta physiol. scand. 1960. 48. 439—442

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Bile Acid Pool in the Rat

Bile Acids and Steroids. 78.

By

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Abstract

ERIKSSON, S. *Bile acid pool in the rat.* Acta physiol. scand. 1960. *48.* 439—442. — The size of the cholic acid pool in the rat has been determined by isotope dilution of the cholic acid in the intestinal tract. A cholic acid pool of about 7 mg per 100 g body weight was found. About 25 % of the cholic acid pool is found in the cecum. The total daily production of unconjugated bile acids in the rat is estimated to about 2.2 mg per 100 g body weight.

In a publication from this laboratory LINDSTEDT and NORMAN (1956) have determined the turnover time of bile acids in the rat by following the fecal excretion of radioactivity after administration of small amounts of labelled bile acids. They found a mean value of 2.3 days (range 1.2—3.1 days) for the biological half life of cholic acid. The half life of chenodesoxycholic acid was of the same order of magnitude. In another paper some preliminary data on the size of the bile acid pool in the rat were reported (ERIKSSON 1957). Using isotope dilution of the bile acids in the small intestine we found a pool size of about 12 mg sodium taurocholate in a 200 g rat. This value was found to agree fairly well with the amount of sodium taurocholate excreted in a bile fistula rat during the first 24 hours after the operation.

NORMAN and SJÖVALL (1958) have found that if a rat is sacrificed 24—70 hours after receiving an intraperitoneal injection of labelled cholic acid the greatest amount of activity remaining in the body is found in the small intestinal contents, 35 % in the large intestinal contents and 9 % in the small intestinal wall. Only 2—4 % of the activity was found in the liver. From these data it is obvious that the main part of the bile acid pool is to be found in the intestine.

NORMAN and SJÖVALL also found that an absorption of bile acids can take place from the cecum and thus the cecal bile acids might be regarded as part of the circulating bile acid pool. Therefore a more detailed study of the size

and distribution of the cholic acid pool in the rat has been made and the results are reported in this paper.

The cholic acid in the intestinal tract, excised from killed animals, has been determined by isotope dilution, a method that offers several advantages. There is no need for quantitative isolation of the cholic acid and its specific activity is not influenced by metabolic reactions occurring in the living animal, *e. g.* the transformation of desoxycholic acid into cholic acid.

Material and methods

Male rats of the institute stock weighing about 200 g were used. They had been given a diet consisting of oats and barley. C^{14}-carboxyl-labelled cholic acid prepared by BERGSTRÖM, ROTTENBERG and VOLTZ (1953) was used in the experiments. It had a specific activity of about 500 cpm per mg when counted in an infinitely thin layer under a Tracerlab TGC 2 G. M. tube.

The animals were killed by a blow on the neck. The small intestine (Exp. no. 1, 2, 3, 4) or the whole intestine (Exp. no. 5, 6, 7) were immediately excised and finely divided in ethanol. Usually three animals were combined in one experiment. The labelled cholic acid (30—40 mg) was added to the ethanol.

The intestines and their contents were then extracted three times for one hour in boiling ethanol. After each extraction the cooled solution was filtered. The combined filtrates were evaporated to dryness *in vacuo* and the residue was distributed between equal volumes (100 ml) of 70 % ethanol and light petroleum by a three stage countercurrent distribution. The ethanol phases were taken to dryness *in vacuo* and the residue was hydrolyzed in 2-N sodium hydroxide at 110° for 6 hours. The solution was then acidified with hydrochloric acid, extracted three times with ethyl ether and the combined extracts washed with water and evaporated to dryness. This material was again distributed between 70 % ethanol and light petroleum to remove remaining fatty acids. The ethanol phases were taken to dryness *in vacuo* and the residue subjected to reversed phase chromatography according to NORMAN (1953). Phase system C of NORMAN was used. In this system a distinct separation of cholic and 7-ketodesoxycholic acids is obtained (NORMAN and SJÖVALL 1958). The fractions containing the cholic acid peak were combined and taken to dryness. The residue was dissolved in sodium carbonate, acidified to pH 1 with hydrochloric acid and extracted three times with ethyl ether. The combined ether extracts were washed with water until free of hydrochloric acid and evaporated to dryness. The residue was repeatedly crystallized from ethyl acetate, ethanol/water and acetic acid/water until the specific activity remained constant.

The amount of cholic acid in the intestinal tract was calculated using the following relationships:

$$A \cdot S_1 = (A + X) \cdot S_2$$

where

A = the amount of C^{14}-labelled cholic acid added.
S_1 = the specific activity of A.
X = the amount of cholic acid in the intestines.
S_2 = the specific activity of the mixture $A + X$.

Thus $X = \dfrac{A(S_1 - S_2)}{S_2}$.

Table I. Intestinal content of cholic acid. The values are expressed as mg per 100 g body weight

Exp. no.	Cholic acid mg/100 g	Sodium taurocholate mg/100 g	Number of animals	Determination performed using
1	4.71	6.2	3	small intestine
2	6.67	8.8	3	» »
3	3.60	4.8	3	» »
4	4.07	5.4	3	» »
5	6.70	(8.9)	3	whole intestine
6	5.10	(6.7)	3	» »
7	7.85	(10.3)	3	» »

Results and discussion

When the bile acids have been excreted into the duodenum the major part is reabsorbed, transported via the portal vessels to the liver and reexcreted in the bile. During each enterohepatic circulation a minor part of the circulating bile acids is lost via the feces. During its passage through the large intestine the bile acid molecule is extensively modified by the intestinal microorganisms; the conjugates are split and further modifications of the free acids occur (NORMAN and SJÖVALL 1958, BERGSTRÖM and NORMAN 1953, LINDSTEDT and NORMAN 1955). By chromatographic analysis of different parts of the intestinal tract after the administration of labelled cholic acid NORMAN and SJÖVALL (1958) have shown that the cholic acid molecule remains intact in the small intestine but as soon as it reaches the cecum profound changes take place.

In Table I are shown the results obtained by the technic described above. The amount of sodium taurocholate present in the small intestine (Exp. no. 1, 2, 3, 4) varies between 4.8 and 8.8 mg with a mean value of 6.3 mg. The values for the whole intestine (Exp. no. 5, 6, 7) vary between 6.7 and 10.3 mg with a mean value of 8.6 mg per 100 g body weight. These results confirm that a part of the cholic acid pool is found in the cecum. According to these figures about 25 % of the total cholic acid pool is found below the small intestine. NORMAN and SJÖVALL (1958) found a somewhat higher value, 35 %, in their studies on the distribution of labelled cholic acid after intraperitoneal injection. However, their figure includes metabolites formed by the action o bacteria on the cholic acid in the cecum.

Of the bile acids present in the cecum the main part thus seems to be present as unmodified cholic acid. The metabolites as well as unmodified cholic acid can be absorbed from the cecum but their rate of absorption is relatively slow (NORMAN and SJÖVALL 1958). The bile acids normally occurring in rat bile are cholic and chenodesoxycholic acids in an approximate propor-

tion of 8 : 2 (ERIKSSON 1957). Allowing for this figure the total circulating bile acid pool in the rat is estimated to about 8 mg per 100 g body weight. When using the term total circulating bile acid pool for this figure we are aware that this figure is probably somewhat low because it does not take into account 1) the amount of bile acids in the liver, bile duct and portal venous system and 2) the amount of bile acids present in the cecum as metabolites. As pointed out above these metabolites can be absorbed from the cecum at a slow rate and thus one should include them in the total circulating or miscable bile acid pool. In spite of these approximations it is reasonable to calculate with a total miscable bile acid pool of about 8 mg per 100 g body weight. If this figure is combined with the mean value for the half life the total daily production of unconjugated bile acids in the rat is found to be about 2.2 mg per 100 g body weight.

PORTMAN and MURPHY (1958) have studied the influence of various diets on the turnover rates and pool sizes of cholic acid in the rat. They found cholate pool sizes of about 100 mg per kg in rats on a Purina chow diet. When synthetic diets were used the cholate pool size diminished to about half this value. However, certain objections can be raised to their method of determining the pool size; they determined the specific activity of cholic acid obtained from a bile duct only 4 hours after the injection of the labelled cholic acid. Probably an equilibrium state between the injected cholic acid and the cholic acid in the intestinal contents cannot be reached in such a short time interval, especially as about 25 % of the cholic acid pool is found in the cecum.

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The Influence of Rest Pauses on Mechanical Efficiency

By

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Abstract

CHRISTENSEN, E. H., R. HEDMAN and I. HOLMDAHL. *The influence of rest pauses on mechanical efficiency.* Acta physiol. scand. 1960. *48.* 443—447. — Two subjects performed a given quantity of work on a Krogh bicycle ergometer within one hour. With a relatively low load the work was continuous, with higher loads breaks of varied length and periodicity were introduced. Mechanical efficiency was the same or slightly less when continuous work was replaced by discontinuous work.

During the later years KARRASCH and MÜLLER (1951) and MÜLLER and KARRASCH (1955) have investigated the influence of work pauses of different length and frequency on fatigue. As an index of fatigue the total number of heart beats above resting level during the recovery period after work (Erholungspulssumme E.P.S.) has been introduced. The following experiments were performed with a similar technique as used by the above mentioned authors but the main interest was concentrated on mechanical efficiency. From a theoretical as well as from a practical point of view it was of interest to investigate to what an extent large changes in work load and varied duration and frequency of work pauses influence the total energy output for a given quantity of work.

Subjects and Methods

The subjects were two well trained students. One was a female, I. H., 21 years old, weight 60 kg and height 174 cm. Her capacity for oxygen intake was 3.2 l/min or 53 ml/kg×min, her observed basal oxygen intake was 0.22 l/min and the basal pulse rate averaged 62. The other subject was a male, R. H., 25 years old, weight 74 kg

and height 177 cm. His capacity for oxygen intake was 4.6 l/min or 62 ml/kg×min, his observed basal oxygen intake was 0.26 l/min and his basal pulse rate averaged 49. The female subject, I. H., had to perform a total quantity of 24,000 kpm and the male subject, R. H., 36,000 kpm within one hour.

Four series of experiments were performed and each one was repeated three to five times to make sure that no training effect should influence the results. In series (I) the work load on the Krogh bicycle ergometer was 400 and 600 kpm/min for the female and the male subject respectively, and the work was carried on for 60 min without rest pauses. In series (II) the loads were 1,000 and 1,500 kpm/min respectively, and 2 min of work were followed by 3 min of rest during the one hour period. In series (III) the loads were the same as in (II) but the periods of work and rest were reduced to 0.5 and 0.75 min respectively. In series (IV) the loads were 500 kpm/min and 750 kpm/min respectively, and rest pauses of 6 min duration were introduced after 24 and 54 min. During the rest pauses the subjects were sitting on the bicycle. The pedal frequency was always 50 rpm. With a work load of 1,000 kpm/min I. H. could work in a steady state for 30 min with only a slight increase in the blood lactic acid concentration, her maximal value was 20 mg per 100 ml. The male subject could do the same at 1,500 kpm/min, with a corresponding value of 30 mg per 100 ml.

The experiments started at 7 or 8 o'clock in the morning with the subjects in basal conditions. The oxygen consumption was determined with the Douglas bag technique. The total amount of expired air was collected during the one hour work period and during recovery. The recovery time lasted from 30 to 50 min beyond the one hour work period. For heart rate measurements an electrocardiographic pulse counter was used and the heart rate was continuously recorded during the one hour and during recovery.

B.M.R. and basal heart rate were determined when the subject had rested on a couch for about 30 min; even during recovery, after the work hour was finished, they rested on the couch placed close to the bicycle ergometer. The room temperature ranged between 16° and 19° C. A small electrical fan, placed at a convenient distance from the subjects, was put on whenever wanted to secure sufficient skin cooling by evaporation of sweat. In this way an attempt was made to avoid a possible increase in pulse rate due to disturbances in heat regulation.

Results and Discussion

Oxygen consumption and mechanical efficiency

In Table I mean values and standard errors of the means for oxygen intake are given. The oxygen consumption during the one hour period exclusive of the observed basal oxygen intake (*i. e.* net oxygen intake) was for I.H. 51.49, 51.73, 52.47 and 50.82 l for series (I), (II), (III) and (IV) respectively. No statistical significant difference between the four series was found. For R.H. the corresponding net oxygen consumption ranged between 73.70 and 78.80 l. A statistical significant difference was found when the series (II) and (III) at 1,500 kpm/min were compared with (I) and (IV) where the lower loads of 600 and 750 kpm/min were used. The difference is however small, amounting to less than 7 per cent. The same results are reached when the total net O_2 intake during 1 h "work period" and recovery are compared (see Table I). The energy cost per kpm of work or the mechanical efficiency is consequently the same or practically the same, whether the work is performed continuously

Table I. Total net oxygen intake during continuous and intermittent work for the two subjects

Series	I continuous work	II 2 min work 3 min rest	III 0.5 min work 0.75 min rest	IV work 2×24 min rest 2×6 min
I. H. ♀ 24,000 kpm	400 kpm/min n = 5	1,000 kpm/min n = 5	1,000 kpm/min n = 4	500 kpm/min n = 4
total net O_2 intake (1) during 1 h "work period"	51.49 ± 0.51	51.73 ± 0.80	52.47 ± 1.12	50.82 ± 0.85
total net O_2 intake (1) during 1 h "work period" and recovery	52.72 ± 0.38	53.24 ± 0.73	53.35 ± 1.56	52.25 ± 1.05
mech. efficiency per cent[1]	22.0	21.8	21.7	22.2
R. H. ♂ 36,000 kpm	600 kpm/min n = 4	1,500 kpm/min n = 4	1,500 kpm/min n = 3	750 kpm/min n = 3
total net O_2 intake (1) during 1 h "work period"	73.70 ± 0.74	[2,3] 78.80 ± 0.56	[3,4] 77.92 ± 0.38	74.16 ± 0.31
total net O_2 intake (1) during 1 h "work period" and recovery	75.47 ± 0.62	[2,3] 80.20 ± 0.55	[3,4] 79.55 ± 0.39	75.39 ± 0.33
mech. efficiency per cent[1]	23.0	21.7	21.9	23.1

[1] The caloric coefficient of oxygen was set to 4.85
[2] Significantly higher than series I, $0.01 > P > 0.001$
[3] » » » » IV, $P < 0.001$
[4] » » » » IV, $0.01 > P > 0.001$

for one hour with an easy load or discontinuously with heavier loads (see Table I).

These results are in agreement with the results of CROWDEN (1934) but hardly with the assumption of MÜLLER and HETTINGER (1957) that pauses of 0.75 min or more should increase the oxygen demand for a following work period significantly compared to the normal steady state level.

At present we are inclined to think that the increased oxygen intake during recovery after a single short spell of work does not allow any definite conclusions as to the actual muscle metabolism during work. Work always means a certain disturbance from basal conditions as also MÜLLER and HETTINGER mention. The hormonal balance, the heat balance etc. will be disturbed, and it is therefore quite understandable that basal conditions are not attained immediately when work stops. We are of course not denying the existence of a true oxygen debt, but think that it might lead to erroneous conclusions if the total increase in oxygen intake during recovery is used for calculating the

Table II. Total number of heart beats during continuous and intermittent work for the two subjects

	I continuous work	II 2 min work 3 min rest	III 0.5 min work 0.75 min rest	IV work 2 × 24 min rest 2 × 6 min
I. H. ♀ 24,000 kpm	400 kpm/min n = 5	1,000 kpm/min n = 5	1,000 kpm/min n = 4	500 kpm/min n = 4
total number of heart beats during 1 h "work period"	6,572 ± 106	6,658 ± 159	6,681 ± 234	6,315 ± 79
number of heart beats above resting level after 1 h "work period"	120 ± 13	307 ± 83	75 ± 26	21 ± 6
R. H. ♂ 36,000 kpm	600 kpm/min n = 4	1,500 kpm/min n = 4	1,500 kpm/min n = 3	750 kpm/min n = 3
total number of heart beats during 1 h "work period"	5,870 ± 109	5,991 ± 98	5,848 ± 93	5,874 ± 67
number of heart beats above resting level after 1 h "work period"	108 ± 26	397 ± 46	257 ± 25	171 ± 11

true oxygen demand and the mechanical efficiency of the metabolic processes that take place during short spells of muscular work.

Our criticism does not only effect the conclusions of MÜLLER and HETTINGER (1957) but also the ones of ASMUSSEN (1946) and of CHRISTENSEN and HÖGBERG (1950).

Heart rate

The total number of heart beats during the one hour "work period" ranged for I.H. between 6,315 and 6,681 and for R.H. between 5,848 and 5,991. Compare Table II. No statistical significant difference was found between any of the series. The marked difference in pulse reaction of the two subjects — the work load for R.H. was 50 per cent higher than for I.H. — corresponds well to the marked difference in aerobic capacity of the two subjects.

If in our experiments the total number of heart beats above resting level after the 1 h work period is taken as an index of fatigue, the results for R.H. agree fairly well with those of MÜLLER and KARRASCH (1955): "Die Ermüdung ist am geringsten, wenn das geforderte Stundenpensum in pausenloser Arbeit bewältigt wird." However, I.H. had the lowest number of heart beats after 500 kpm/min with two rest pauses of 6 min each, and no significant differences were found between series (I) and (II), or between (I) and (III). For the

high work loads of 1,000 and 1,500 kpm/min, respectively, the lowest number of heart beats for both subjects is found in series (III) with short spells of work (0.5 min) and rest (0.75 min). This result is in agreement with those of MÜLLER and KARRASCH (1955).

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Intermittent Muscular Work

By

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Abstract

ÅSTRAND, I., P.-O. ÅSTRAND, E. H. CHRISTENSEN and R. HEDMAN. *Intermittent muscular work.* Acta physiol. scand. 1960. *48.* 448—453. — The physiological effect of rest pauses on a non-steady state work (2,160 kpm/min) was studied. A physically well trained subject performed in one hour a total amount of 64,800 kpm on a bicycle ergometer by intermittent work with 0.5, 1, 2 or 3 min periods of work and rest. Total O_2 intake, total pulmonary ventilation, total number of heart beats and blood lactic acid concentration during the work hour and during recovery were determined. It was found that the heavy work when split into short periods of work and rest (of 0.5 or 1 min duration) was transformed to a submaximal load on circulation and respiration and was well tolerated during one hour. With longer periods (of 2 or 3 min duration) the work output got close to the upper limit of performance and could be fulfilled only with the utmost strain. These findings are discussed from a physiological and practical point of view. In order to explain the low lactic acid values during the short periods of work and rest it was proposed that the myohemoglobin has an important function as an oxygen store during short spells of heavy muscular work.

It is a well known fact that the oxygen intake during the initial period of heavy work does not correspond to the energy demand; due to a time lag in respiration and circulation a certain oxygen deficit arises. It takes one or several minutes, depending upon the work load and the physical fitness of the individual, before the oxygen intake reaches a steady state level. At severe work a steady state will never be reached, and the work time will be limited by anaerobic metabolites in the muscles, blood and other tissues.

The problem for our present research work is to analyze the effect of rest

pauses on different physiological functions responsible for the increased metabolism during such a non-steady state work (2,160 kpm/min).

In these experiments the work periods and the pauses varied between 0.5 and 3 min. During the same experiment they were always constant and of equal length. The total work time was one hour and the effective work time and the rest time were consequently always 30 min each. During the one hour the total work amounted to 64,800 kpm, or an average of 1,080 kpm/min. In this way it was possible to compare the physiological effect of intermittent work of 2,160 kpm/min and continuous work of 1,080 kpm/min. The trained subject could perform the latter work for hours without fatigue.

If the periods for work and rest are 0.5 min, 60 initial work periods will occur with all the consequences this may have as far as O_2 intake, heart rate, pulmonary ventilation and so forth are concerned; if the periods are 3 min the number of initial periods will only be 10, and the different physiological functions responsible for O_2 transport may, towards the end of the work period, reach values that are fairly close to the demand. Consequently one might expect to find a reduced tendency for anaerobic metabolism in experiments with 3 min periods compared to the shorter ones in which the oxygen transport during work always will be far below the demand. The following experiments, however, gave the opposite result.

Subject and Methods

All experiments were done with one physically well trained male subject, R. H., age 25 years, weight 74 kg and height 177 cm. His capacity for oxygen intake at 6 min of work on the bicycle ergometer was 4.6 l/min or 62 ml/kg×min. His basal pulse rate averaged 49 beats per min and his basal O_2 intake was 0.26 l/min.

Work was performed on a Krogh bicycle ergometer at 60 pedal revolutions per min with a load of 6 kg corresponding to 2,160 kpm/min; in the experiments with continuous work for one hour the load was 3 kg and the work load was 1,080 kpm/min. The expired air was collected in Douglas bags and analyzed according to the Haldane technique. The heart rate was recorded with an electrocardiographic pulse counter during the work hour and during 60 min of recovery after work. The resting values for pulse rate and O_2 intake, and the recovery values were taken with the subject reclining on a bed close to the bicycle. The determinations during work pauses were done with the subject sitting on the bicycle. Blood samples for lactic acid determination were taken from the warmed up finger tip and the analyses were done according to Barker and Summerson (1941) with the modification of Ström (1949). Rectal temperature was determined before and immediately after work, as was the weight ($\pm$ 50 g) to get information about the heat regulation.

Results

Certain of the investigated functions are shown in Table I to make possible a comparison between different work forms. The number of experiments is limited, motivated partly by the high accuracy of the methods used and partly by the extreme demands which a work form of 2 or 3 min places upon the

Table I. Total O_2 intake, work efficiency, total number of heart beats and total pulmonary ventilation during continuous and discontinuous work

	I total O_2 intake (l) "work hour" STPD	II total O_2 intake (l) "recovery hour" STPD	III work efficiency per cent	IV total number of heart beats "work hour"	V total number of heart beats recovery hour"	VI total pulm. vent. (l) "work hour" BTPS
continuous work 1,080 kpm/min	145.5	19.9	23.4	7,904		2,847
1,080 kpm/min	145.9	19.1	23.4	7,859	3,683	2,916
discontinuous work 2,160 kpm/min						
work min / pause min						
0.5 / 0.5	154.1	21.8	21.5	8,637	4,299	3,266
0.5 / 0.5	154.2	19.6	21.9	8,493	4,276	3,202
1 / 1	152.2	21.7	21.6			3,330
1 / 1	152.4	20.0	21.9	8,295	4,211	3,406
2 / 2	160.1	21.0	20.4	8,579	4,715	3,908
3 / 3	162.9	24.2	19.4	9,215	5,219	4,355

subject when the work shall be carried on for one hour. However, further experiments were made although only some of the functions given in the table were measured, and these results are in agreement with the values in the table. Furthermore, it should be pointed out that each result given in Table I on O_2 intake and pulmonary ventilation during work and recovery is based on a large number of determinations in one and the same experiment. For example, the values for O_2 intake at 1,080 kpm/min are based upon a total of 38 determinations made between the 2nd and the 60th min of work. An average for O_2 intake of 2.44 l/min, with a standard deviation of $\pm$ 0.038 l/min and an error of the mean of $\pm$ 0.006 l/min, illustrate the accuracy of the method and the stability of the subject.

From Table I it can be seen that the mechanical efficiency is highest (23.4 per cent) at continuous work of 1,080 kpm/min; at discontinuous work with 0.5 or 1 min periods the mechanical efficiency is 21.7 per cent, at 2 min periods, 20.4 per cent and at 3 min periods, 19.4 per cent. The moderate lowering of the efficiency at the short periods as compared with the 2 and 3 min periods is also illustrated by the difference in the total number of heart beats (IV and V). The pulse sum increased from approximately 11,500 at continuous work to about 12,500 with the one min periods and to about 14,400 with the 3 min periods. The total pulmonary ventilation (VI) shows the same tendency;

Table II. Maximal and minimal values of O_2 intake, pulmonary ventilation, heart rate and blood lactic acid concentration during continuous and discontinuous work

	O_2 intake l/min STPD		pulm. vent. l/min BTPS		heart rate beats/min		lactic acid mg per 100 ml
	max	min	max	min	max	min	
continuous work 1,080 kpm/min	2.44		49.0		134		12
2,160 kpm/min 9 min	4.60		124.0		204		150
discontinuous work 2,160 kpm/min							
work min / pause min							
0.5 / 0.5	2.90	2.30	62.5	44.5	150	137	20
1 / 1	2.93	2.23	65.3	47.5	167	99	45
2 / 2	4.40	1.00	95.0	35.0	178	106	95
3 / 3	4.60	1.00	107.0	36.0	188	118	120

it increased from 2,880 l during the work hour at continuous work to 3,370 l at work with 1 min periods and to 4,350 l at the 3 min periods, which means an increase of 17 and 51 per cent respectively. With regard to heat regulation, work at 3 min periods seems to differ from the others, with an increase in the rectal temperature of 2.0° C (38.9° C). In the other cases, the rectal temperature after the work hour was around 38° C, with a maximal increase of 1.35° C. Loss of weight was more or less identical for the different forms of work and amounted to about 700 g.

Work with short periods was subjectively felt to be relatively light, and the subject experienced no fatigue after one hour. Work at 2 and specially at 3 min periods meant a nearly maximal or a maximal load. Only by strong motivation could this work be performed for a whole hour. A closer analysis of the values in Table II, which contains maximal and minimal values for O_2 intake, pulmonary ventilation and pulse rate for intermittent work, provides an explanation for this difference in subjective strain. The values for continuous work at 1,080 and 2,160 kpm/min respectively, are also included in Table II for comparison. The maximal values refer to determinations made during the last half minute of the work period. In the same way, the minimal values refer to the last half minute of the rest period. It should be pointed out that due to the technique used for collection of expiratory air during 0.5 min, the given figures do not represent the absolute maximal or minimal values; this may have a certain significance at the 0.5 and 1 min periods. Since the high values for O_2 intake and pulse rate are first reached 15 to 20 min after the beginning of the work hour, the values given in Table II are representative for the latter part of the work period and not for the first 5 or 10 min. The lactic acid values

are valid for the time immediately following the end of the work hour, but generally the blood lactic acid concentration reached a more or less constant level about 15 to 20 min after the beginning of the work hour; a continuous accumulation of lactic acid consequently did not take place.

The most surprising results obtained were the low lactic acid values, about 20 mg per 100 ml, at the work with short periods. According to the introductory considerations, this work form might be expected to result in relatively unfavourable conditions for oxygen supply to the active muscles. The low lactic acid values found contradict this assumption.

Discussion

The results given above are of interest for several reasons. They confirm findings reported elsewhere by CHRISTENSEN, HEDMAN and HOLMDAHL (1960) that the mechanical efficiency at intermittent work, with suitable load and duration of work and rest periods, does not lie on a considerably lower level than at continuous work.

The fact that one can obtain a great amount of work done at an extremely heavy load with a clear submaximal load on circulation and respiration by suitable application of short work and rest periods, is of great practical and physiological interest.

The results illustrate that one can divide the total amount of work into suitable periods in such a way that one can induce training of large muscle groups without simultaneously loading the respiratory and circulatory organs (work with short periods over long time). By choosing longer periods, for example 2 to 3 min, one can obtain a high training effect also on respiration and circulation. This is of interest not only for the training of sportsmen, but also for rehabilitation of patients during the convalescent period, etc.

The reason why older workers in spite of lowered capacity for oxygen intake, to a surprisingly high degree, remain in physically heavy jobs such as forestry and farming may also be explained. If these workers spontaneously choose a suitable length of work and pause periods, the acute loads on respiration and circulation do not need to exceed the moderate range corresponding to the old individual's reduced capacity. If, however, the work pace is determined by a machine, even a less heavy work with relatively long work periods may involve an elimination of the older workers.

In the present investigation the work periods and the rest pauses were always equal in duration in the same experiment. Therefore it is difficult to decide whether the short work periods or the short pauses cause the favourable results obtained. This problem will be more thoroughly analyzed in a following investigation.

A relationship between the time for work and for rest of 1 : 1 seems to give practically full recovery if the duration of the period is 0.5 min. On the other hand, this is not at all the case if the period length is 2 or 3 min. It is important

to stress the fact that the so called rest allowances used in industry to avoid overloading of the workers, based on average caloric consumption during the 8-hour work day, according to our findings may have entirely different physiological effects dependent on the duration of work and rest periods.

In order to explain the low lactic acid values at the short periods, two hypotheses may be proposed. The first postulates that the rate of formation of lactic acid is the same, independent of the length of the period, but that lactic acid during the short periods of rest is eliminated almost at the same rate. According to LEHMANN (1953 p. 49) many short rest pauses should imply a more favourable recovery than longer, but thereby fewer, pauses.

The other hypothesis assumes that the formation of lactic acid during the short work periods is reduced to a minimum. This would mean that the liberation of energy during the initial phase (0.5 min) of a work of 2,160 kpm/min could take place practically aerobically. This conflicts with earlier assumptions but seems nevertheless to give the most probable explanation for the experimental findings related here. Oxygen transport by the blood to the muscles during work is both absolutely and relatively less during the short work periods; if in spite of this the work is aerobic, this must depend upon the amount of oxygen which the muscles dispose of at the very moment when the work is started. The oxygen bound to myohemoglobin and to hemoglobin in the muscles and the amount which comes to the muscles with the blood during the 0.5 min of work must be the prerequisite for this aerobic work. During the pauses, even if they are only 0.5 min long, the myohemoglobin must certainly have time to be reloaded with oxygen before the next work period begins. During work periods of 2 or 3 min, the oxygen transport to the muscles is relatively greater but never becomes adequate, and the oxygen bound to myohemoglobin, which suffices for only a short part of the whole work period, becomes of much less importance.

If this assumption is correct, myohemoglobin has a fundamentally important function in addition to the traditionally accepted one, *i. e.* that it plays a certain role as an O_2 buffer in the muscle, being re-charged during relaxation so that it can be reduced during the following contraction. According to the viewpoints presented here, myohemoglobin should represent an oxygen store which is used during the initial phase of work before circulation and respiration are able to reach the values which correspond to the actual oxygen demand. Further research on these points will be taken up in another investigation.

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Acta physiol. scand. 1960. 48. 454—460

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Myohemoglobin as an Oxygen-Store in Man

By

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Abstract

ÅSTRAND, I., P.-O. ÅSTRAND, E. H. CHRISTENSEN and R. HEDMAN. *Myohemoglobin as an oxygen-store in man.* Acta physiol. scand. 1960. *48.* 454—460. — The aim of the present research was to investigate further the possible rôle of myohemoglobin as an oxygen-store during the initial stage of muscular work. One subject worked intermittently with a work load of 2,520 kpm/min with varied duration of work and rest pauses on a bicycle ergometer. A highly significant difference in the blood lactic acid concentration during the experimental time of 30 min was found, at work with short work periods (10 sec, lactic acid concentration about 10—20 mg per 100 ml) compared with relative long ones (60 sec, lactic acid concentration 110—140 mg per 100 ml). The conclusion was drawn that the first type of work is performed aerobically. The calculated oxygen demand, during the work period of 10 sec, however, does not correspond to the measured oxygen intake. A deficit of about 0.43 l O_2 for each period of work will occur. It was suggested that this amount of 0.43 l O_2 is supplied to the working muscle mainly from oxymyohemoglobin. This store function of myohemoglobin is discussed in relation to the present findings and to the results mentioned in the literature.

In an earlier investigation concerning intermittent heavy work (2,160 kpm/min) it was shown that blood lactic acid concentration remains low if work alternates with rest pauses every half minute. If work and rest periods are increased to 3 min the lactic acid concentration will reach high values and the total work time will be limited due to exhaustion (ÅSTRAND *et al.* 1960).

A possible explanation for the aerobic work metabolism when 0.5 min periods are used was given by the assumption that the oxygen bound to the myohemoglobin plays an important rôle in the supply of oxygen to the working muscles in the initial stage of work. With increasing duration of the work

period the relative importance of this oxygen fraction diminishes, and if the transport of oxygen is insufficient for the local demand, anaerobic processes have to cover a certain fraction of work metabolism, and lactic acid accumulates in the muscles and in the blood.

The present work was planned to further elucidate the rôle of myohemoglobin in this respect. To obtain more conclusive results the work load was increased to 2,520 kpm/min. Furthermore, the periods of work and rest were varied independently of each other so that more decisive results as to the relative importance of the rest pauses could be obtained.

Methods

Work was performed by one male, physically well trained subject, R. H., on a Krogh bicycle ergometer. The subject and the methods used were the same as in the earlier experiments (ÅSTRAND *et al.* 1960). The work of 2,520 kpm/min corresponds to a load of 6 kg with 70 pedal revolutions per minute. The total experimental time was, if possible, 30 min. The work periods were always of the same duration throughout one experiment. They lasted for 10, 15, 30 or 60 sec. The rest pauses lasted from 20 up to 240 sec (compare Table I). Due to the different duration of rest pauses the total quantities of work produced on the different experimental days ranged between about 6,000 and 38,000 kpm, and the average work load for the 30 min varied between some 200 kpm/min and 1,260 kpm/min.

Results

Table I summarizes the experiments done and gives the maximal values for blood lactic acid concentration, the total quantity of work performed in the 30 min experiment and other calculated values of importance for the discussion. The total quantity of 25,200 kpm of work will be represented in all series, I—IV arranged according to the duration of the work period in the table, and the quantity of 15,120 kpm will be found in three of the sections. This makes a direct comparison possible. If the work period lasts for 10 sec, 420 kpm will be produced at every work occasion; with longer duration this quantity increases and reaches, at 60 sec, 2,520 kpm. The calculated oxygen demand for these different work quantities are given in the table. These values are, of course, approximate but still they give a good illustration of the varying demands, which are 0.9 l of O_2 for the 10 sec periods and 5.6 l for the 60 sec periods. The calculated values are based upon a mechanical efficiency of 23.0 per cent and a caloric coefficient for oxygen of 4.85.

Fig. 1 and 2 illustrate the changes in blood lactic acid concentration during work with a total quantity of 25,200 and 15,120 kpm respectively. The relationship between work and rest time is always 1 to 2 in Fig. 1, and 1 to 4 in Fig. 2. With the short work periods of 10 sec followed by pauses of 20 sec, the blood lactic acid concentration was about 20 mg per 100 ml, while in the experiment with 40 sec pauses it was approximately 15 mg per 100 ml; that is, in both cases

Table I. Peak blood lactic acid concentration in experiments with intermittent work of various duration of work and pause periods. Work production, effective work time and oxygen demand have been calculated and tabulated

Series	I		II		III					IV		
work periods, sec	10		15		30					60		
pause periods, sec.....	20	40	30	180	30	60	120	180	240	120	180	240
kpm produced in each work period	420		630		1260					2520		
total production in 30 min, kpm	25,200	15,120	25,200	5,800	37,800	25,200	15,120	10,840	8,320	25,200	18,900	15,120
effective work time in 30 min experiment, min	10.0	6.0	10.0	2.3	15.0	10.0	6.0	4.3	3.3	10.0	7.5	6.0
O_2 demand in each work period, l	0.9		1.4		2.7					5.6		
maximal values for blood lactic acid conc. mg per 100 ml..	23	17	31	29	78	70	56	58	41	142	114	114

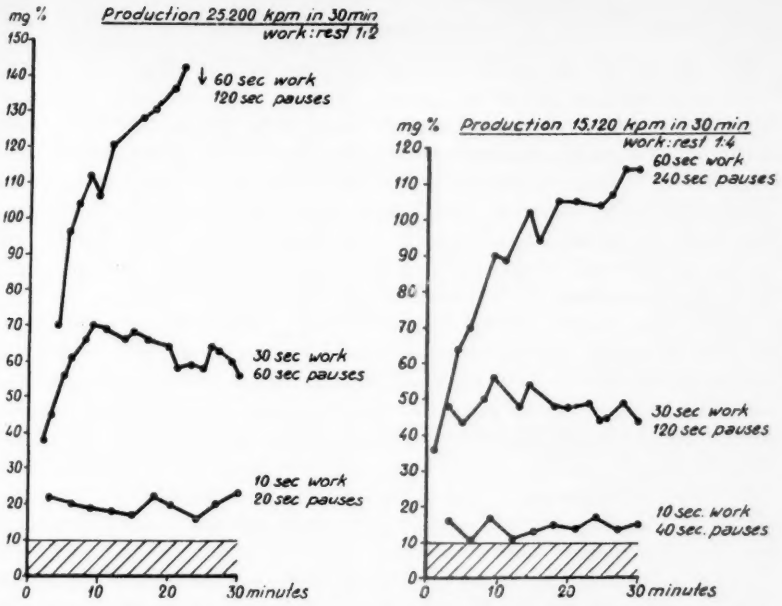

Fig. 1 and 2. The blood lactic acid concentration at a total work production of 1) 25,200 kpm and 2) 15,120 kpm during an experimental time of 30 min. The work is performed with a load of 2,520 kpm/min. The work periods last for 10, 30 and 60 sec and the corresponding rest periods for 1) 20, 60 and 120 sec and for 2) 40, 120 and 240 sec.

very close to the normal rest value of about 10 mg per 100 ml. If the work periods are lengthened to 30 sec and the pauses to 60 sec, one finds a significant increase in the blood lactic acid concentration; after about 9 min a maximal value of 70 mg per 100 ml is reached (see Fig. 1). After that there is a small reduction and the values remain approximately at 60 mg per 100 ml until the end of the experiment. At the longer pauses of 120 sec, and consequently a smaller amount of work performed (Fig. 2), a maximal value is also obtained after about 9 min, now at 56 mg per 100 ml, and thereafter the values are stabilized between 40 and 50 mg per 100 ml. Therefore, even with work periods of 30 sec a certain equilibrium is reached between the production and the elimination of lactic acid. With work periods of 60 sec, however, a corresponding balance is never reached; the lactic acid concentration increases until the end of the experiment. With the relatively short pauses of 120 sec (Fig. 1), the blood lactic acid concentration reached a maximal value of 142 mg per 100 ml after 22 min. The experiment was then interrupted because the subject was no longer able to continue. With pauses of 240 sec (Fig. 2) the task could be fulfilled for 30 min and the blood lactic acid concentration reached a value of 114 mg per 100 ml at the end.

Discussion

The present results confirm earlier findings by ÅSTRAND *et al.* (1960). They show conclusively that a principal difference exists in man's reaction at intermittent work to short and relatively long work periods, even if the total amount of work in a certain time is the same. Furthermore, the results answer the question, whether the length of the work period or the length of the pause is the deciding factor for the blood lactic acid concentration. In the earlier series of experiments, as stated above, the length of the work periods and pauses were always equal in the same experiment, which brought about difficulties for making a conclusive interpretation in this respect. It can be seen from the results in Table I that the most decisive factor is the length of the work period (compare series I—IV). From the results in series III it is most apparent that even the length of the pause has a certain significance. In this series the work period was 30 sec and the pauses on the different experimental days varied between 30 and 240 sec. With a pause of 30 sec there were 30 work occasions during the half hour, with a pause duration of 240 sec there were only 7 work occasions. Fewer work periods naturally decrease the possibilities to produce lactic acid, and longer pauses provides greater possibilities for the elimination of lactic acid. That explains the decrease in blood lactic acid concentration from 78 mg per 100 ml, which was the maximal value with 30 sec pauses, to 41 mg per 100 ml with 240 sec pauses. Accordingly, the duration of the pauses has a secondary importance in comparison to the duration of the work periods. This is also illustrated by the results given in Fig. 1 and 2.

It is of great interest whether or not the present results are in agreement with the hypothesis mentioned above that the difference in the reaction at short (10 and 15 sec) and long (30 and 60 sec) work periods can be explained by the rôle of myohemoglobin as an oxygen-store.

One knows that the oxygen transport by the blood to the working muscles increases with the duration of the work period, and that equalization between oxygen need and supply can take several minutes to occur. In the series of experiments performed with 10 sec of work and 20 sec of rest, the oxygen intake was determined during the work period, and corresponds to 2.80 l/min. During the 10 sec of work the actual oxygen intake was 1/6 of this, or 0.47 l. The oxygen intake during work was 10 times greater than a corresponding rest value of 0.043 l. The oxygen intake during the last 30 sec of a 60 sec work period with 120 sec pauses gave a maximal value of 4.08 l O_2/min, or 0.68 l O_2 for 10 sec. In the latter case the oxygen intake was 16 times greater than the rest value. To be adequate for a load of 2,520 kpm/min an oxygen supply of 5.6 l/min or 0.9 l per 10 sec is required according to Table I. During the experiment with work for 10 sec and pauses for 20 sec we must calculate with a deficit of $0.90 - 0.47 = 0.43$ l O_2. With work for 60 sec and pauses for 120 sec, the oxygen intake during the whole work period was maximally 3.26 l

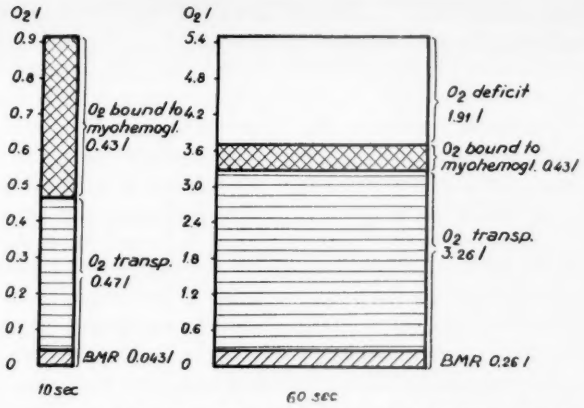

Fig. 3. The oxygen demand for 10 and 60 sec work with a load of 2,520 kpm/min. An attempt is made to illustrate the fraction of O_2 that is a) bound to myohemoglobin, b) transported by the blood and c) O_2 deficit.

(during the first half min 2.43 l/min and during the second half 4.08 l/min). This means a deficit of 5.60 — 3.26 = 2.34 l O_2. The reason that the oxygen intake during the first half minute of a 1 min work period is relatively smaller than during the 10 sec periods (2.43 and 2.80 l/min respectively) is that during the short pauses of 20 sec the circulation and respiration never decline severely before the work is begun again. If the pauses are lengthened to 2 min, the time for adjustment becomes significantly increased.

With work for 10 sec and pauses for 20 sec we must assume practically aerobic conditions in the working muscles. If such were not the case, the 60 work occasions should have brought about a successive accumulation of lactic acid as a consequence, compare Fig. 1. We believe that the conclusion can be drawn that approximately 0.43 l O_2 have been available in the working muscles at the beginning of each new work period, naturally even at the 60 sec periods. Quantitatively this means, that a supply of oxygen for the 10 sec periods is assured by that amount, which is already in the muscles and by the amount which can be transported by the blood during the work itself. For 60 sec work, a deficit of 1.91 l arises. This must be covered by anaerobic processes, which results in an increase of the lactic acid concentration in the blood.

Fig. 3 illustrates schematically the relative importance of the postulated amount of oxygen in the muscles at 10 sec and 60 sec work periods respectively.

Naturally, the oxygen supply to the muscles becomes smaller at a single work occasion, and the anaerobic factor is of greater importance quanti-

tatively. If the work time is sufficiently short the amount of oxygen bound to myohemoglobin should, however, be able to play a decisive rôle for the muscle metabolism even in those cases. A revaluation of the so-called Simonsen-effect must be the consequence. According to MÜLLER and HETTINGER (1957) this effect is important during the first 10 sec of work and results in an extra oxygen consumption as a result of the anaerobic conditions in the muscles.

On the basis of the results given here the amount of the oxygen which is available at the beginning of the work can not, of course, be determined. If one attempts to calculate the amount of oxygen bound to myohemoglobin, one is immediately confronted by a whole series of more or less unknown factors. One is not familiar with the size of the active muscle mass, the myohemoglobin concentration of the musculature or the degree of reduction of oxymyohemoglobin. If one uses the values given in the literature for myohemoglobin for example by BIÖRCK (1949, p. 131), one finds that each gram of muscle can bind about 10 mm^3 O_2. If one assumes 20 kg of active muscles for the subject in the work mentioned here, one arrives at a value of 200 ml O_2. There is still a deficiency of about 230 ml, according to the above. The values given by BIÖRCK are in this case too low, since it is generally accepted that the amount of myohemoglobin increases with training, and the values given above are not derived from specially well trained individuals.

It is quite evident from the investigations of SCHOLANDER, IRVING and GRINNELL (1942) on diving seals that myohemoglobin can constitute an important factor for oxygen supply to the musculature. According to our conception, the experimental results laid forth here indicate that myohemoglobin has an important function as an oxygen-store even in man. The reader is referred to BIÖRCK (1949, p. 42), regarding references for and against such a conception based on earlier findings.

Before a definite answer can be given to the question of the quantitative rôle which myohemoglobin plays in this respect, further investigations are required on myohemoglobin concentration in trained individuals. It is our hope that the experimental results related here will help to create greater interest into this field of research.

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Intracellular Potential Measurements from the Ciliary Processes of the Rabbit Eye in vivo and in vitro

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Abstract

BERGGREN, L., *Intracellular potential measurements from the ciliary processes of the rabbit eye in vivo and in vitro.* Acta physiol. scand. 1960. *48.* 461—470. — The ciliary processes in the rabbit's eye were explored by microelectrodes. On penetrating the epithelial layers with the electrode two well defined levels of potential difference were measured between the penetrating electrode and the external reference electrode. In experiments conducted in vitro the first potential step was about — 30 mV and is suggested to appear when the electrode penetrated the unpigmented cell layer. The second step is suggested to appear when the electrode penetrated the pigmented cell layer. The measured potential difference between the electrodes was — 60 mV during this step. At further penetration it was found that the stroma was nearly equipotential with the external fluid. These potential differences disappeared when the preparation was kept in a potassium free solution and when it was poisoned by cyanide.

A small number of successful experiments in vivo confirmed the findings made in vitro.

The ciliary processes take part in the formation of aqueous humour and might be regarded as continuously working glands. The anatomy of the processes is unique, since the transport of blood constituents must take place across two different epithelial cell layers in series. In this investigation the membrane potential of these different cells has been measured in the rabbit eye.

Anatomy

Fig. 1 gives a general view of a ciliary process (in a human eye). (See further SALZMANN 1912, LAUBER 1936, WOLF 1940, HOLMBERG 1959.) Two epithelial cell layers line the stroma of the process. *The pigmented layer* is separated from the stroma by a cuticular lamina. The latter forms shallow sockets for the cells of the epithelium.

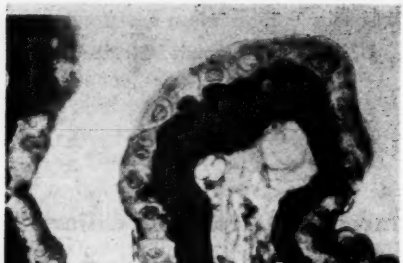

Fig. 1. Ciliary process from a human eye (after Lauber).

It must not be mistaken for the basement membrane which is a fine submicroscopic structure. The pigment epithelium represents the forward continuation of the pigment layer of the retina. The cells are filled with pigment granules and are about 10—15 μ in height. Posterior to the processes the height of the cells increases. *The unpigmented layer*, the ciliary epithelium, is continuous at the ora serrata with the nervous layer of the retina. It is firmly attached to the pigment epithelium. The cells have a height of about 10 μ, increasing posterior to the processes to about 30 μ. In the forward continuation of the cell layers onto the back side of the iris even the non-pigmented cells begin to be filled with granules. The side of the non-pigmented epithelium facing the posterior chamber is intimately connected to the membrana limitans interna ciliaris, which in its turn serves as the attachment of the zonules.

Microscopic examination of the ciliary processes in rabbits showed coarsely analogous conditions. There was a tendency for both epithelial layers to decrease in height on the top of the processes. Large variation in cell height (from 5—20 μ) could, however, be seen between different processes in the same preparation. Electron microscopy shows that the inner limiting membrane has a fibrillar structure (PAPPAS and SMELSER 1958). As a consequence of this, it might perhaps not be a limiting barrier to the passage of secreted material into the posterior chamber.

Aqueous humour formation

It has been suggested that the ciliary processes take part in the secretion of aqueous humour. This view has partly been based on histological observations; viz. similarities to other secretory glands, the occurrence of Nadi-oxidase etc. Both epithelial layers have been singled out as the site of formation, the pigment layer (TREACHER COLLINS 1891) as well as the non-pigmented layer (SEIDEL 1937 inter alia). Recent histologic investigations (ROHEN 1954) have been interpreted as indicating that both layers take part in the secretion process.

Chemical analyses of the components of aqueous and blood, and investigations of the accumulation and turnover rates, proved that at least some components of the aqueous must be secreted (see reviews by DAVSON 1956 and LANGHAM 1958). There

is evidence that sodium enters the posterior chamber in a one way direction (KINSEY and PALM 1955), but further details in the transport system seem still to be unknown.

Electromotive forces in the ciliary process

LEHMANN and MEESMANN (1924) and GRANT (1956) measured a potential difference of about 10 mV between the anterior chamber (positive) and the jugular vein. FISCHER (1932) found a potential difference of similar magnitude between the aqueous humour and the stroma of the iris.

Indirect evidence for a potential between the ciliary epithelium and stroma was presented by FRIEDENWALD and STIEHLER (1938). The selective transfer of acid and basic dyes across freshly excised ciliary processes in albino rabbits was taken to indicate that an electromotive force brought about movement of ions. The source of energy was supposed to be a difference in oxidation-reduction potential between epithelium and stroma. Measured with indicators the redox-potential of the epithelium was found to be + 100 mV and the redox-potential in the stroma — 130 mV. A reversible oxidation-reduction system between the epithelium and stroma was assumed to pass electrons from stroma to epithelium. The redox pump hypothesis has long formed a basis for theories of the secretion of aqueous. However, the existence of a difference in redox-potential does not necessarily imply the existence of a true potential difference (USSING 1949).

Moreover, stoichiometrically, the oxygen consumption in corpus ciliare (DE ROETTH 1953) is not sufficient to maintain transport of sodium or chloride by a redox pump (discussed by BERLINER and BALLINTINE 1956).

It has not been possible to separate the individual contributions of the two epithelial layers to the secretory processes in the formation of aqueous humour. In an analysis of the transport processes not only the potential across the whole "barrier" between aqueous and stroma is of interest, but it seems important and necessary to know the different potential levels within the "barrier". The aim of the present investigation has been to measure the potentials across all the individual membranes between aqueous humour and stroma.

Material

Male rabbits, weighing about 2 kg, and fed on a diet of hay, oats and water ad lib. were used, in the in vitro experiments albino rabbits only, in the in vivo experiments both albino and pigmented animals.

Methods

Preparation

The animals were anesthetized with an intravenous injection of Numal® "Roche" (allylisopropyl barbituric acid) in a dosage of 60 mg/kg body weight. Additional topical anesthesia, consisting of two drops of a two per cent solution of Xylocain® "Astra" (lidocain) was sometimes used, but not in the in vivo measurements.

In vitro experiments. The preparations for the in vitro experiments were made in the following way. The eye was enucleated and a circumferential incision about 2—3 mm posterior to the limbus was made. The posterior side of the ciliary body was then carefully cleaned from adhering vitreous body, lens, strands of the lens capsule and zonulae. Thereby also the limiting membrane was at least partially removed. The

membrane can be easily recognized for it appears as a cast of the ciliary processes suspended from the zonular fibres. Four peripheral radial cuts were made through the ocular coats in order to flatten the specimen. It was then attached to a plexiglass support. The preparation was kept immersed in a large volume (500 ml) of Krebs solution.

In vivo experiments. For the in vivo experiments the iris and the ciliary body were transplanted from one eye to the other eye of the same rabbit. This was done in order to allow access to the ciliary processes from the anterior chamber. The transplantation was made in the following way. One eye was enucleated, and a 3 mm wide strip of iris together with ciliary body was gently teased free from the scleral spur. The other eye was then opened by a corneal incision with a cataract knife. The iris-ciliary body preparation was gently introduced into the (opened) anterior chamber with the anterior side of the iris facing the host iris. The preparation was kept in Krebs solution while outside the body. The ciliary body of the preparation thus remained free from contact with any tissue of the host eye. No corneal suture was made. The eye was dressed with Aureomycin® eye ointment "Lederle" (chlortetracyclin) and the eye lids gently closed. After a week the eye generally was without irritation and the transplant began to be vascularized from the host iris. There were many failures with this technique. For example the transplanted iris tended to envelop the whole transplant, or the transplant became embedded in the host iris. There were, however, also some successes where the iris portion of the transplant became attached to the host iris, while the ciliary body remained free except at its root, and the transplants were satisfactorily vascularized from the host iris. Injection of fluorescein intravenously showed the dye in the transplanted ciliary body and it was later seen to come out from the transplant and accumulate in the anterior chamber.

Two months after the operation the animals were used for potential recordings. They were anesthetized in the manner described. For further deepening of the anesthesia ether was used.

The head of the animal was then immobilized with a head holder mounted on a cross-feed as described by WERSÄLL (1958). Eye movements were restricted by sutures in the extraocular muscles and the eye lids. The cornea was then trephined over the transplant. The preparation was moistened with isotonic NaCl solution and covered with petroleum jelly in order to reduce evaporation. A microelectrode was then applied to the transplant with a micromanipulator and a reference electrode was inserted into an extraocular muscle.

Recording

Potential differences were measured between a large reference electrode and a KCl (3M) filled glass microelectrode (NASTUK and HODGKIN 1950, BROCK, COOMBS and ECCLES 1952, TASAKI, POLLEY and ORREGO 1954), with a tip diameter of about 1 μ and a resistance of 15—50 megohm. The recording instrument consisted of a Philips pH-meter PR 9400 connected to a Honeywell-Brown strip chart recorder with a paper speed of 1 or 4 inches per minute. The PR 9400 has a vibrating condensor input working at 50 c/sec and an input impedance of $10^{12}\Omega$ at d. c.

The microelectrode was moved with the aid of a micromanipulator. The vertical movement was measured to the nearest 5 μ. Vertical movement was either made manually or automatically, in the latter case with a speed of 200 μ/min.

Nomenclature. Potential differences are given as potential of microelectrode minus potential of reference electrode, *i. e.* as inside potential minus outside potential.

Solutions

The standard medium used was Krebs solution no. 1 made up from stock solutions according to Krebs (1950) and with the following composition:

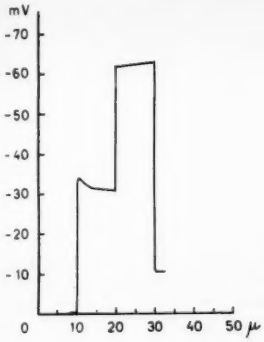

Fig. 2. Potential recording with continuous movement of the microelectrode with a speed of 200 μ/min. Abscissa = distance in μ, ordinate = potential difference in mV. Exp. no. 470.

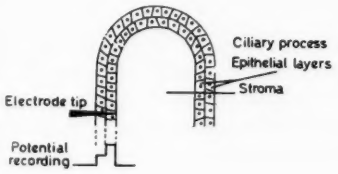

Fig. 3. Sketch of ciliary process and potential recording.

NaCl	103 mM	
KCl	5 mM	
$CaCl_2$	3 mM	
$MgSO_4$ 7 H_2O	1 mM	
KH_2PO_4	1 mM	
$NaHCO_3$	27 mM	
Na-pyruvate	5 mM	(Theodor Schuchardt, München)
Na-fumarate	6 mM	» » »
Na-L-glutamate	5 mM	(Merck, Darmstadt)
glucose	13 mM	

The pH of the solution was 7.4 and was checked with a glass electrode. The temperature of the preparation was kept at desired values, within $\pm$ 0.5° C, with the aid of a thermostat. The solution was intermittently bubbled through with a gas mixture containing 93.5 % O_2 and 6.5 % CO_2.

Results

In vitro experiments

When the microelectrode approached the epithelium of a ciliary process a sudden change appeared in the potential difference between the two electrodes. Further advancement of about 20 μ revealed a second step in the potential difference and a still further advancement revealed a third potential step (Fig. 2 and 3). Advancement after this third potential step revealed a region of about 100—200 μ of approximate equipotential while still further on three more steps were observed at about 20 μ distance from each other.

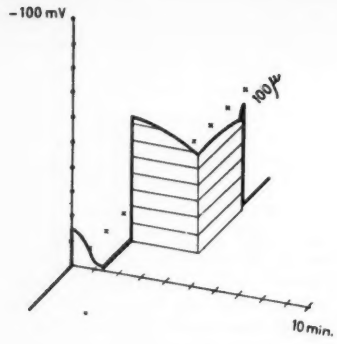

Fig. 4. Three-dimensional figure of the stability of the potentials, discontinuous movement. Abscissa = time in minutes, ordinate = mV. At right angles to these the third axis shows depth of penetration. Exp. no. 719.

The sudden steps in measured potential seemed to be strikingly similar to those observed when a microelectrode penetrates a muscle or nerve fibre or a nerve cell. It seemed therefore safe to conclude that the sudden steps occurred when the electrode penetrated the cell membranes, and that the amplitudes of the steps were a measure of the membrane potentials of the cells.

When these measurements were compared with the histology of the ciliary processes it was apparent that: a) the first step was associated with the penetration of the membrane between the outside (ciliary) epithelial cell and the outside solution, b) the second step was associated with the penetration of the membranes between the outside and inside (pigment) epithelial cells, c) the third step was associated with the penetration of the membrane of the inner cell towards the stroma, d) the 100—200 μ region of equipotential was within the stroma and e) the three more steps were associated with the penetration of the two cell layers from the stroma towards the outside solution.

When these main features of the measurement had been established, *i. e.* that the two potential steps within the epithelial layers represent the polarization or membrane potential of the two cell layers, it was tried to obtain reasonable figures for the value of these membrane potentials. The electrode might not always penetrate the cell without causing a substantial damage. Almost all penetrations gave a sudden potential step but the recorded potential always decreased in amplitude with time, indicating that the cells were seriously damaged by the penetration of the electrode (Fig. 4). Because of this, a large number of penetrations had to be done. Only those measurements were taken into account where the potential step was sudden and the new potential level was well defined. All the other measurements were neglected since they seemed to be misleading due to the damage caused by the penetration. Moreover only potentials from the ciliary processes were taken into account. On the back side of the iris similar but smaller potential levels were measured. In Table I are the measurements presented from those penetrations

Table I

	N	Mean mV	standard deviation mV	s. e. of mean mV
V_1	28	— 28	± 7.9	± 1.5
V_2	28	— 60	± 8.2	± 1.6
V_3	28	— 4	± 4.8	± 0.9
V_2—V_3	28	— 56	± 8.9	± 1.7

V_1 is the potential measured during first step, *i. e.* membrane potential of external cell layer. V_2 is potential during second step, *i. e.* membrane potential of internal cell layer relative to outside solution. V_3 is potential during third step, *i. e.* potential of stroma relative to outside solution. V_2—V_3 is potential of inside cell layer relative to potential of stroma.
N = number of eyes. The single eye is treated as the statistical unit. Thus if several successful recordings were obtained in the same eye the mean of these recordings was taken as representative of this eye.

that were considered reliable. The membrane potential of the external (ciliary) cells was thus about — 30 mV while that of the internal (pigmented) cells was about — 55 or — 60 mV and the stroma was at nearly equipotential (about — 5 mV) relative to the external fluid.

Now the question arises whether the difference of 25 to 30 mV between the membrane potential of the two cell layers is characteristic for the two types of epithelial cells, or whether any systematic errors in the experiment possibly could account for this difference. The external (ciliary) cells did as a rule run down appreciably quicker than the internal (pigmented) cells: the first potential level decreased about 10 % in 0.5 to 1 minute and disappeared within 2 to 3 min after penetration with the electrode left untouched within the cell, while the second potential level decreased about 10 % in about 1 to 5 min and disappeared in about 20 min. The external cells were thus clearly more sensitive to damage than the internal cells. It seems, however, highly doubtful that this difference could account for more than a fraction of the difference in measured membrane potential between the two cell types.

Effects of changes in experimental conditions

The temperature of the preparation had no appreciable influence on the value of the membrane potentials in the range tested (20 — 36° C), neither had the pH of the Krebs solution any marked effect on it in the range between pH 7.2 and 8.0. At lower values (< 7.2) the membrane potentials decreased strikingly. Cyanide tested in concentrations > 1 mg/ml decreased membrane potentials very effectively, after about 15 min in the cyanide solution membrane potentials of only a few mV were measured.

A potassium free Krebs solution, where potassium was replaced by sodium

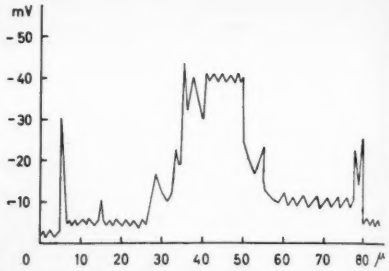

Fig. 5. Potential recording from in vivo experiment. Continuous manual movement of the microelectrode, much slower than in fig. 2. Exp. no. 195.

Fig. 6. Section of a transplanted ciliary body in a rabbit. The two epithelial cell layers are clearly seen.

had a remarkable effect, since no membrane potentials more negative than — 10 mV were found even after a reasonably short time (about 2 min) in the potassium free solution. Only 25 penetrations on 6 eyes were made. The effect was so striking that it is anyhow held for certain.

Results from in vivo experiments

The original experimental group comprised 20 animals (10 albino rabbits and 10 pigmented rabbits). Slit lamp microscopy after the operation revealed 12 successful cases of transplantation (5 albino rabbits and 7 pigmented rabbits).

Acceptable recordings were obtained from 3 animals (1 albino rabbit and 2 pigmented rabbits). The remaining rabbits were rejected because of difficulties in keeping the transplant immobile during the penetration of the microelectrode.

The results from experiments conducted in vitro were confirmed. Three potential levels were established here too. The distance between the points where the potential steps occurred amounted to 5—20 μ. The first level was maximally — 30 mV with a mean of — 21 mV and the second maximally — 43 mV with a mean of — 30 mV. The stromal potential was about — 5 mV. (See fig. 5.)

Fig. 6 is a section of a transplanted ciliary body demonstrating the two layers of ciliary epithelium.

Discussion

It was consistently found that transversal penetration of the ciliary epithelial layers revealed a potential record where the potential changed suddenly in three well defined steps. The distance between the points where the steps occurred was about 20 μ with extreme values of 5 and 50 μ. All the findings are in favour of the view that the changes in potential appeared at the moment when the electrode penetrated the cell walls, *i. e.* the measurements are measurements of the membrane potential in the ciliary epithelial layers. These membrane potentials 30 mV respectively 60 mV in the two cells were somewhat lower than, although of the same order of magnitude as the membrane potential in some other structures (frog muscle 85 mV, frog nerve about 70 mV, squid nerve about 65 mV etc). The potential difference between the aqueous humour and the stroma of the ciliary processes was about — 5 mV and this value is in good agreement with the earlier measurements of the potential difference between blood and aqueous humor of — 6 to — 10 mV (LEHMANN and MEESMANN 1924).

It is evident that an oblique penetration is expected to give a more complicated potential record when the electrode penetrates a whole series of cells. This was verified in some experiments where penetration was made oblique.

The measurements in the in vivo experiments are qualitatively in full agreement with the above conclusions. The measured potential steps were here somewhat smaller, but this might be insignificant since it is conceivable that the transplanted ciliary body still was somewhat disturbed in its function.

The experiments have so far shown that it is possible to measure the membrane potentials in the cells in the two well defined layers of the ciliary processes. The two epithelial layers are histologically continuous with the epithelial layers of the iris. It is rather remarkable in view of this, that the membrane potential in the epithelial cells of the iris was distinctly smaller than in the ciliary processes. The mechanism behind the membrane potential remains still unknown. The same can be said about the role of the membrane potential in ionic transport across the epithelium. It may, however, be pointed out that the membrane potential decreased very rapidly (compared with peripheral nerve) when the structure was poisoned with cyanide and further, attention should be given to the very surprising effect of a potassium free medium. Although the mechanism for these effects remains obscure they might give a clue for further investigations.

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Acta physiol. scand. 1960. 48. 471—478

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Immuno-electrophoretical Investigations of Mouse Serum Protein Fractions Obtained by Ammonium Sulphate Fractionation

By

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Abstract

CLAUSEN, J. and J. HEREMANS. *Immuno-electrophoretical investigations of mouse serum protein fractions obtained by ammonium sulphate fractionation.* Acta physiol. scand. 1960. *48.* 471—478. — This study contains data on 21 mouse serum proteins demonstrated by immuno-electrophoretical analysis of normal F_1 (CBA X DBA/2) mice. Supernatants and precipitates obtained with ammonium sulphate at concentrations ranging from 1.2 to 2.8 M were investigated immuno-electrophoretically, and the precipitation range of most of the components was established. Data regarding the physico-chemical properties of several mouse proteins are given as a basis for comparison with some of the better known serum proteins.

Among the numerous methods developed in recent years to characterize the proteins which constitute the serum protein spectrum, GRABAR's immunoelectrophoresis appears to have the highest resolving power (GRABAR and WILLIAMS 1953). So far more than 25 distinct proteins have been described in human serum by means of this method. Recently HEREMANS, CLAUSEN, HEREMANS and RASK-NIELSEN (1959) have published immuno-electrophoretic tracings of normal mouse serum. Twenty-one different mouse serum proteins were differentiated by this method and the proteins in the beta- and gamma-region were described. In the present report all serum proteins are characterized by means of their solubility in ammonium sulphate solutions of increasing molarity, and finally an attempt has been made to establish some parallels to human serum proteins with similar properties.

Materials and Methods

Animals

The mice used for these experiments were healthy adult F_1 hybrids between CBA and DBA/2 strains. Blood was collected by decapitation following ether anesthesia. Several blood samples were pooled in a centrifuge tube and after clotting serum was separated from the clot by centrifugation at 6,000 rpm. In this manner hemolysis could be completely avoided.

Immuno-electrophoresis

The micromethod of SCHEIDEGGER (1955) somewhat modified by HEREMANS was used (HEREMANS *et al.* 1959 a).

Antisera

Antisera against normal mouse serum were obtained as described by HEREMANS *et al.* 1959 a.

Salting-out procedure

Ammonium sulphate concentrations, ranging from 1.2 to 2.8 M, were used. Before addition of the salt solution serum was diluted to double the volume with distilled water, to reduce interference from absorption and co-precipitation phenomena. To obtain the desired final salt concentration in a final volume of 2.0 ml a neutralized 4.0 M ammonium sulphate solution was added to the serum-water mixture in appropriate proportions. The mixtures were incubated for 24 hrs. in a water bath at 37° C and centrifuged at 18,000 rpm for 20 min. The compact precipitates were washed several times with the same concentration of ammonium sulphate solutions as used for precipitation and again isolated by centrifugation. They were finally redissolved in sodium-barbital-HCl buffer (pH 8.5, ionic strength 0.1 M) making the volume of the solution the same size as that of the original serum sample.

Semi-quantitative estimation of serum proteins

It is not possible to determine the serum proteins observed in immuno-electrophoresis quantitatively, the change in the position of the precipitation line caused by an increased concentration of a given protein being too small to allow such a determination. Moreover the amount of precipitate formed by the antigen-antibody reaction does not indicate the amount of antigen because the precipitate is soluble in excess of the antigen.

However, immuno-electrophoresis allows semi-quantitative determination by means of two methods:

1) *Estimation of the intensity and extent of the precipitation line and of its distance from the antibody reservoir* may provide information with respect to the amount of protein. For instance, if the albumin bow in an ammonium sulphate precipitate is indistinct, only localized to the intermediate part of its normally occurring locus and situated more medially than normally, this indicates that a small amount of this protein is present.

2) *The dilution method.* When successive dilutions of serum are investigated immuno-electrophoretically, it is possible, particularly as regards the main components, to estimate the weakest dilution in which these proteins can be seen in the tracings. This dilution is a measure of the relative concentration of the protein.

Results

In the terminology suggested in our previous description of the mouse serum proteins (HEREMANS *et al.* 1959 a) conventional names such as alpha-1, alpha-2 etc. are used. The different proteins which constitute each of these areas are further designated as I, II, III etc. according to their decreasing mobilities. The designation beta-3-area is only used to indicate that in the immuno-electrophoretic tracings we have found some proteins between the slow gamma and the beta region with protracted precipitation lines extending into the beta-2-region and superimposing upon the gamma-precipitation line.

A. The serum proteins in immuno-electrophoresis

A given antiserum does not show all the serum protein fractions. For this reason the following description of the 21 proteins observed so far, does not apply to a single tracing but is the result of tracings from several immuno-electrophoreses developed with different antisera, as for instance with NBD, GT and L_{80} antisera (Fig. 1).

The following proteins, listed according to decreasing mobility, have been found:

1) One prealbumin.

2) One albumin, the main component.

3) Four alpha-1-proteins alpha-1-I . . . . IV, with the anodical half of their lines located in the anodical half of the albumin bow. The alpha-1-I is presumably the main component.

4) Six alpha-2-proteins called alpha-2-I . . . . VI. Alpha-2-I and alpha-2-II are the two main components in this region. They could easily be distinguished in the dilution 1:8, in which dilution all the other precipitation lines in this area had disappeared. Alpha-2-III takes up specific lipid stains. The mobility of this protein was variable depending on the batch of agar gel used.

5) One beta-1-protein called beta-1-I, usually poorly developed.

6) Four beta-2-proteins: beta-2-I . . . . IV, of which beta-2-I is the major component in this region.

7) Three beta-3-proteins: beta-3-I . . . . III. Beta-3-I has a double-bent curvature. Beta-3-II and beta-3-III are characterized by their protracted lines.

8) One gamma-globulin, also a straight and protracted line, the anodal part of which is often superimposed upon the beta-3-lines.

The proteins in the beta- and gamma-regions have been described in detail in a previous publication (HEREMANS *et al.* 1959 a).

B. Ammonium sulphate fractionation

Mouse serum was submitted to salting-out precipitation with 1.2 to 2.8 M ammonium sulphate solutions. All precipitates and supernatants were examined immuno-electrophoretically.

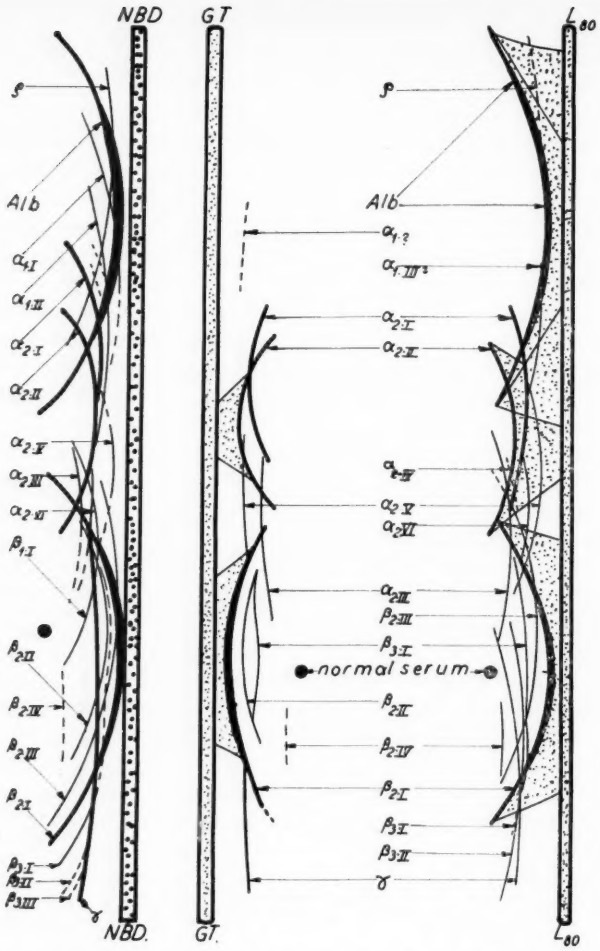

Figure 1. The precipitation lines in immuno-electrophoresis of normal mouse serum, developed with three different antisera.
Left: immuno-electrophoresis developed with the standard polyvalent antiserum NBD. The majority of the optimally observed lines are seen.
Central: immuno-electrophoresis developed with the weak antiserum GT; only few lines are visible.
Right: immuno-electrophoresis developed with the polyvalent antiserum L_{80}. Numerous lines are seen, among them alpha-2-III and alpha-2-V which are not well developed with antiserum NBD.

The high resolving property of immuno-electrophoresis is unfortunately associated with a superimposition of the precipitation lines in the alpha and

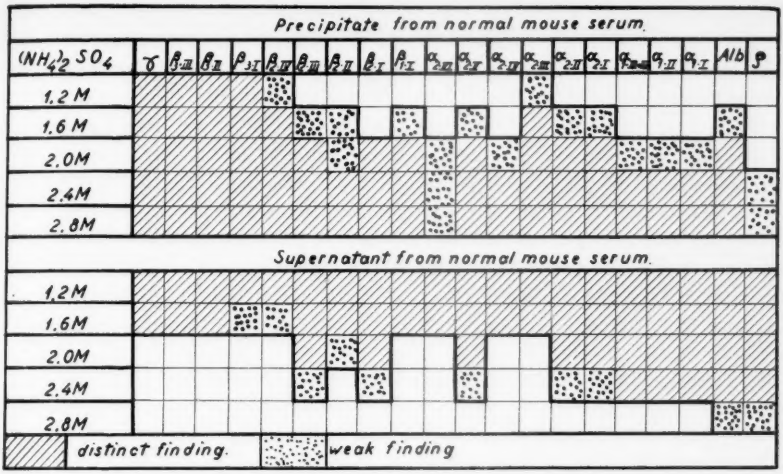

Figure 2. Immuno-electrophoretically determined protein fractions in the precipitates and supernatants from ammonium sulphate fractionation of pooled normal mouse serum.

beta areas, which causes difficulties in the exact identification of each of the lines in the series of precipitates and supernatants when using one polyvalent antiserum only. It was therefore necessary to use several antisera, containing different antibodies against normal mouse serum. As seen from the results indicated on fig. 2 none of the salt fractions were immunologically pure when submitted to immuno-electrophoresis.

The proteins found were:

1) A gamma-globulin group of proteins comprising gamma-, all three beta-3-, beta-2-IV and alpha-2-III globulins, all of which were completely cleared from the supernatant above 1.6—1.8 M ammonium sulphate concentrations. These proteins have marked euglobulin properties and flocculated out from the serum upon dialysis against distilled water for 24 hrs.

2) A group comprising beta-2-III, beta-2-II, beta-2-I, beta-1-I, alpha-2-V, alpha-2-II and alpha-2-I, all of which begin to flocculate out at 1.6 M (beta-2-I at 1.8 M), and are all completely precipitated at 2.0 to about 2.4 M.

3) Two proteins alpha-2-IV and alpha-2-VI both of which are represented by weak precipitation lines, possibly due to their low serum level or to their poor antigenicity or both. These two proteins precipitate late, at about 2.0 to 2.4 M, and they were cleared from the supernatant at 2.4 M.

4) All alpha-1-globulins precipitate only above 2.0 M and were cleared from the supernatant at 2.8 M.

5) Albumin and prealbumin were found throughout the whole series of supernatants, and traces were still soluble at 2.8 M.

Discussion

So far 21 proteins have been observed in immuno-electrophoresis of normal mouse serum.

All four of the alpha-1-globulins and two of the three beta-3-globulins (beta-3-II and beta-3-III) do not resemble proteins in human serum, when the mobilities and the groupings are used as indicators. The alpha-2-I and alpha-2-II globulins, the main components in the alpha-2-area, are probably rich in carbohydrate; as in paper-electrophoresis this area is stained intensely in the P. A. S.-staining procedure (CLAUSEN, RASK-NIELSEN and GORMSEN 1957). However, no definite conclusions concerning these fractions can be drawn from a comparison with human serum, where the whole alpha-2-group is composed of glycoproteins (SCHULTZE *et al.* 1955).

The salting-out behaviour and euglobulin properties of the slow lipoprotein, called alpha-2-III, are reminiscent of the human slow lipoprotein system (URIEL and GRABAR 1956).

The alpha-2-V globulin is a pseudoglobulin. Immuno-electrophoresis of mouse sera in experimental amyloidosis (CLAUSEN, CHRISTENSEN and RASK-NIELSEN 1959) revealed a rise in the alpha-2-V globulin levels, in cases where this condition was associated with a nephrotic syndrome. In man a considerable increase is seen in an alpha-2-macroglobulin in the nephrotic syndrome. This protein, usually referred to as "Schultze's alpha-2-macroglobulin" (SCHULTZE *et al.* 1955) contains carbohydrate and is easily precipitated by ammonium sulphate. It is tempting to identify the mouse protein alpha-2-V with human alpha-2-macroglobulin. This is probably justified by the marked increase in the bound carbohydrate of the alpha-2-region observed in experimental amyloid nephrosis (CLAUSEN *et al.* 1959 a).

The identification of beta-2-I mouse globulin with the iron binding component of mouse serum has been described in another report (CLAUSEN *et al.* 1959). Its salting-out properties are quite similar to those of human transferrin (SCHULTZE, HEIDE and MÜLLER 1956).

The beta-2-III globulin has revealed no physico-chemical property which allows correlation of this protein to any known protein in human serum; nevertheless, attention has been drawn to this protein because of its specific biological behaviour, appearing as changes observed during malignant growth and following conveyance of foreign proteins (CLAUSEN *et al.* 1959, RASK-NIELSEN, GORMSEN and CLAUSEN 1959, CLAUSEN *et al.* 1959 a, CLAUSEN *et al.* 1959 c).

The beta-3-I globulin with a double-bent bow follows the gamma system in its salting-out properties. It is also a pronounced euglobulin. A similar protein is seen in human serum. It has been isolated and found to possess the activity of the third component of complement (MÜLLER-EBERHARD and NILSSON 1959).

The identical salting-out properties of gamma-beta-3-II-beta-3-III-

globulins corroborates the relationship between these components. The precipitation lines of all three proteins are protracted lines extending far anodically and their partial superposition and coalescence makes their individual resolution impossible as is the case with the gamma-beta-2-A lines in human tracings (HEREMANS, HEREMANS and SCHULTZE 1959). They seem to represent what could be called a "gamma-system", analogous to the gamma-beta-2-A system in the human, or to the gamma-T system in the horse (WUNDERLY 1958).

A salt fractionation procedure is not a good basis for the isolation of the immuno-electrophoretically pure mouse serum proteins. A similar limitation was mentioned by WILLIAMS and GRABAR (1955) in the case of human serum. Nevertheless, such fractionations may be of limited use, when only partly selective absorptions of certain antibodies from a given antiserum are desired. For instance, absorption of a polyvalent antiserum with the 2.0 M supernatant was used to clarify the immuno-electrophoretic picture in a study of experimental amyloidosis (CLAUSEN *et al.* 1959 a).

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